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THE BRITISH JOURNAL

OF

EXPERIMENTAL PATHOLOGY

THE ÆTIOLOGY OF RICKETS: AN EXPERIMENTAL INVESTIGATION.—PART II.

D. NOËL PATON AND ALEXANDER WATSON.

From the Institute of Physiology, University of Glasgow.

Received for publication April 2nd, 1923.

In our previous papers (1918, 1921) we adduced evidence that (1) milk fats and their associated vitamin A have no significant influence in preventing the onset of rickets, or in curing the condition in young dogs; (2) that pups may be reared on separated dried milk and bread without developing the condition; and (3) that pups kept in the open air grow up free of rickets on a diet poorer in milk fats than that of members of the same litter kept in the laboratory which developed the disease. The explanation of these last results is probably to be found in the action of sunlight in preventing and curing rickets and in the influence of temperature and more rapid cooling upon the metabolism.

In their recent paper Powers, Park and Simmonds (1922) state:

"When the expression 'exposure to direct sunlight' is used, it is inclusive of all that is ordinarily indissolubly associated with sunlight exposure. Sunlight itself, the effect of changes in the atmosphere by solar radiation, temperature, out-of-door air, these and other factors unknown to us, acting singly or in combination, may be responsible for the results usually attributed simply to 'sunlight.'* These experiments bring out, above all else, that exposure to direct sunlight and out-of-door air promotes in a very wonderful way the health, bodily vigour and longevity of animals which otherwise are unable to adapt themselves to markedly adverse environmental conditions.

"Sunlight probably exerts no specific anti-xerophthalmic influence, but acts by raising the level of the cellular activity of the organism to a point where the progress of the disease is held in check or allowed to advance very slowly and with relatively little disturbance."

Our conclusions as regards the want of any action of milk fats is supported by the work of others, although Mellanby's (1919) experiments were accepted by the Vitamin Committee of the Medical Research Council (1919) as demon-

* "Experiments are now in progress which seem to indicate that out-of-door air does have a favourable influence upon rats fed on a rickets-producing diet."

strating their anti-rachitic action. One of us (Paton, 1922[2]) has elsewhere analysed his results and has shown that they do not warrant this conclusion.

The results of Hess, McCann and Pappenheimer (1920–21), and those of Shipley and Park and their colleagues (1921), indicate that butter-fat has no anti-rachitic action, either prophylactic or curative. Sherman and Pappenheimer (1920–21), and Pappenheimer, McCann and Zucker (1922[1]) showed that rats may be reared upon a diet apparently free of any source of vitamin A if sufficient basic sodium phosphate is supplied.

McCollum, Simmonds, Becker and Shipley (1922) found that after oxidative destruction of the vitamin A of cod-liver oil, the residue manifests an anti-rachitic action, but no influence upon xerophthalmia.

Pappenheimer, McCann and Zucker (1922[2]) also have shown that vitamin A in butter or butter-fat neither prevents nor cures rickets.

Zucker, Pappenheimer and Barnett (1922) found that the constituent of cod-liver oil which acts in rickets is in the unsaponifiable fraction of the oil. More recently Zucker (1923) states that it may be extracted by acetone from the calcium soaps and that it is 1000 times as active as the original oil.

That the substance, or substances, in cod-liver oil which, according to McCollum and others, regulates the deposition of bone-salts plays at most a secondary part is shown by the fact that innumerable children and pups grow up free of rickets without the use of cod-liver oil.

The results of these various investigators strongly suggest that any beneficial action which milk may possess on calcification of bone is due rather to the proportion of calcium and phosphorus contained than to the presence of a vitamin.

The fact that sunlight or ultra-violet rays will prevent the onset of rickets upon a diet on which, without them, the disease would appear, is a further indication that the action of these hypothetical vitamins is not essential to the prevention of rickets—that rickets is not a disease due to the deficiency of a vitamin. The hypothesis that the action of sunlight on the skin is to form such specific vitamins seems somewhat far-fetched, and is opposed to the experimental evidence adduced by Hume (1922) and Goldblatt and Soames (1922).

The part played by the adequacy or inadequacy of the supply of calcium and of phosphoric acid in producing ricket-like changes in the bones has recently received considerable attention, chiefly in America (Park, 1923).

The outcome of this American work is to demonstrate firstly the self-evident fact that the limitation of either of these substances leads to the failure of proper ossification, and secondly, to show that the changes at the epiphyses resemble those seen in rickets. How far it is justifiable to identify such a condition with rickets, as seen in children, is, perhaps, questionable. To show that certain morphological changes may be produced by an alteration in the conditions does not necessarily mean that similar changes are always due to these alterations.

True rickets certainly may develop upon an adequate intake of calcium and of phosphorus, and if it is due to a defective supply of these elements to growing bone, it must be ascribed either to decreased absorption from the gut or to the transport of one or other, or both, in unsuitable form in the

blood (Paton, 1922[1]). So far there seems no evidence of a defective capacity to fix lime and phosphoric acid in the osteogenic tissue. Grosser's (1920) observations that the retention of calcium may be increased in rachitic children by the subcutaneous administration of calcium glycerophosphates seem to show that the power of fixing the inorganic elements is retained.

The evidence adduced by Telfer (1922–23[2]) seems to indicate that decreased absorption may produce ricket-like changes, but leaves open the question of whether this is the causal factor in the production of rickets.

Whether the action of cod-liver oil and of sunlight in favouring the retention of calcium and phosphorus is upon the absorption by the gut, or upon the metabolic processes by which the elements are fixed, has never been investigated. Our argument (Findlay, Paton and Sharpe, 1921) that the absence of a marked decrease in the calcium of the blood in rickets indicates that failure of absorption of calcium is not the cause of the condition is perhaps unwarranted. The maintenance of a definite concentration of calcium ions in the blood and tissues seems to be essential to normal metabolism, and, even if absorption were interfered with, the stored calcium of bone, or the calcium which ought to go to bone, may be retained in or passed to the blood to maintain that concentration.

That the inorganic P_2O_5 of the plasma is generally reduced in amount has been shown by Howland and Kramer (1921), but to argue from this that the failure of ossification is due to a limitation of necessary P_2O_5 is not justifiable till we have further knowledge of the significance of the normally larger amount of P_2O_5 in organic combination which exists in the blood. The decrease in the inorganic P_2O_5 of the plasma may merely be an accompaniment of some altered metabolism of the phospho-lipoids (Goto, 1922).

It is quite possible that the inorganic P_2O_5 is simply an excretion, as indicated by Meigs, Blatherwick and Cary (1918). This view is supported by the increase of inorganic phosphates which occurs in the plasma in uræmia (de Wesselow, 1923).

McCollum has emphasised the point that it is not so much the absolute amounts of CaO and P_2O_5 in the diet which matters, but rather their proportion, and it is possible that an undue proportion of CaO may lead simply to an increased restriction to the gut of P_2O_5 as phosphate of lime, as has been indicated by Telfer (1922–3[1]), especially on a phosphorus-poor diet.

An increase in the alkalinity of the intestinal content might decrease the absorption of lime and phosphoric acid, and Zucker, Johnston and Barnett (1922) have recently shown that a decrease of the acid base ratio may lead to the development of ricket-like changes in the bones of rats. This is merely an extension of a long series of observations since Seeman's work in 1879 (see Keith and Forbes, 1914).

Telfer's work indicates what Schabad (1910) had previously suggested—that the error of phosphorus metabolism in rickets may be of even greater importance than the disturbance in the metabolism of calcium.

PRESENT INVESTIGATION.

The present paper is concerned with the following questions:

1. The relative importance of restriction of the intake of calcium and of

vitamin A in the production of ricket-like changes in the skeleton; and the retention of calcium and of phosphoric acid on a calcium-rich and calcium-poor diet.

2. The question of how far the action of olive oil in favouring the onset of rickets is due to the absence of a vitamin.

3. The influence of subdued light in favouring the onset of rickets in pups.

4. The possibility of rickets having an infective origin.

(1) *The Relative Importance of Restriction of Calcium and of Vitamin A in the Production of Rickets; and the Retention of Calcium and of Phosphoric Acid on a Calcium-rich and Calcium-poor Diet.*

E. Voit (1880), long before any idea of the possible existence of vitamin A had been suggested, maintained that rickets could be produced in growing dogs by feeding on a diet poor in calcium. One pup was fed upon lean horse flesh with bacon, the other upon the same diet with the addition of bone ash. The former developed rickets, the latter did not, although the supply of vitamin A in bacon fat and in lean meat is now generally accepted to be negligible.

A *résumé* of the more recent work upon the effects of restricted calcium intake is given by Korenchevsky (1922). He states:

"Therefore I think that a careful examination of the question must lead to the conclusion that in some cases it may be possible by calcium starvation to produce a disease which clinically, chemically, macroscopically and even histologically may simulate rickets. In spite of this similarity, I am convinced that there is an essential difference between the two, and I consider the introduction of the term 'rickets from calcium starvation' (but not pseudo-rachitic osteoporosis) as most desirable. In this group of rickets I propose to include the skeletal changes caused by calcium starvation, or those caused by the introduction of strontium into the organism of animals in a state of calcium starvation. In the latter case there is no osteoporosis, and therefore the term pseudo-osteoporosis is inapplicable."

He concludes that—

"Other forms of rickets may be produced on a diet containing a normal amount of calcium."

This our previous experiments upon pups has clearly shown to be the case.

The investigations already recorded seem to indicate that the onset of rickets may be retarded by giving an adequate supply of calcium, even when the supply of vitamin A is negligible—that the disease is not necessarily due to deficiency of vitamins either in milk or in cod-liver oil. This conclusion is further supported by the recent work on pigs by Elliot, Crichton and Orr (1922).

A re-investigation of the effects of varied calcium intake with more careful metabolic observations using the more reliable methods of analysis which are now available seemed necessary, since the results of previous investigators are so contradictory (Korenchevsky, 1922).

In our experiments the intake of calcium and of vitamin A was reduced to a minimum by the use of bread and lean meat as a diet. The calcium amounted to only about 0·02 grm. per kilo per day as CaO, while the requirements of a young child, presumably less than those of the more rapidly growing dog, are about 0·15 to 0·2 grm. per kilo. In other pups the calcium

supply was made adequate by the addition of calcium lactate to raise the intake of CaO to 0·15 to 0·2 grm. per kilo. In all the experiments the supply of phosphoric acid (P_2O_5) was more than adequate—about 0·12 to 0·17 grm. per kilo.

Experiment X.—This experiment was started when the pups were somewhat too old—8 weeks—and it was of too short duration to yield conclusive results, but it seemed to show that in the absence of any further supply of vitamin than that afforded by lean beef and beef-fat, a pup may develop a normal skeleton for ten weeks on an intake of only 0·02 grm. of CaO per kilo per day.

Three collie pups of one litter which had been on a diet of full-cream milk (dried) and bread were, at 8 weeks old, put in pens on a concrete floor in a room without direct sunlight, and had the following diets, which were increased, to keep the intake per kilo roughly the same, as the pups gained weight.

TABLE I.—*Diet of Pups (weights in grms.).*

			Total.	Per kilo.
Pups 1 and 2:				
Full-cream dried milk .	33	Fat .	7·36 .	1·96
		CaO .	0·566 .	0·15
Bread . .	45	Calories.	348 .	92·8
Pup 3:				
Lean beef . .	54	Fat .	14·17 .	2·71
Bread . .	100	CaO .	0·10 .	0·02
Beef-fat . .	9	Calories.	574·6 .	110·0

TABLE II.—*Percentage of CaO and of Fat in Foods used.*

	CaO.	Fat.
Bread . .	0·08 .	0·77
Dried milk .	1·59 .	21·0
Beef . . .	0·04 .	8·16

The growth-curves are given in Chart 1*. No signs of rickets developed. X-ray photographs taken after 7 weeks showed no abnormality in the bones.

The experiments terminated after ten weeks, the dogs being 18 weeks old and having passed the age at which rickets develops. A limitation of lime to 0·02 grm. CaO per kilo of body-weight per day failed to produce the changes described by Voit after eight weeks.

Experiment XV.—The object of the experiment was to test the effects of restricted calcium intake, with and without the addition of vitamin A in butter-fat, in the absence of direct sunlight,

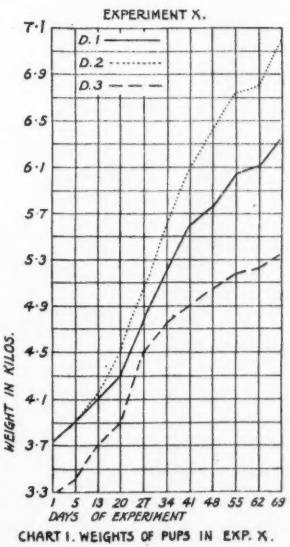

CHART 1. WEIGHTS OF PUPS IN EXP. X.

* In the charts of the growth-curves the day of the experiment is given in the abscissa and the weights in kilos in the ordinates.

and to study the calcium and phosphoric acid balance on a calcium-poor and calcium-rich diet.

Four mongrel pups of the same litter (1 and 3 developed as collies, 2 and 4 as whippet-like dogs) at 7 weeks of age were put in pens, six feet square, in a room without direct sunlight, and had the following diets, which were increased as shown below to maintain the intake per kilo at nearly the same level.

TABLE III.—*Diets* (*in grms.*).

	Sept. 27th. All diets.	Oct. 19th. All diets.	November 1st.		Nov. 10th. All diets.
			1 and 3.	2 and 4.	
Bread, dry	60	75	85	75	85
Meat	50	60	60	60	70
Butter	3	4	—	4	—

The weight curves are shown in Chart 2. The caloric value of the diet per kilo was between 100 and 130.

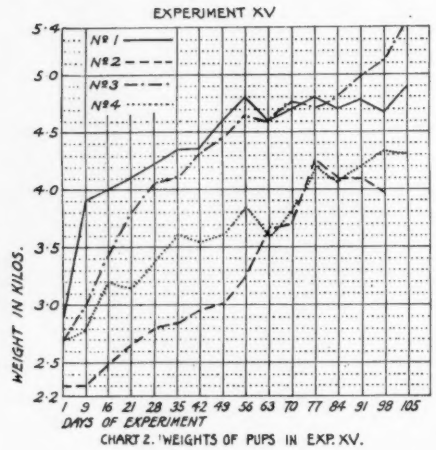

CHART 2. WEIGHTS OF PUPS IN EXP. XV.

After four weeks the diets of pups 2 and 4 contained 2·7 grm. of fat per kilo, and those of pups 1 and 3, 1·4 grm. per kilo. Pups 2 and 3 each had 3·3 grm. calcium lactate (0·6 grm. CaO) per day in addition to the CaO of the food-stuffs (0·068 grm.), *i. e.* they had 0·668 grm. in all per day at the beginning, and 0·96 grm. per day at the end of the experiment. Pups 1 and 4 had 0·068 grm. per day at the beginning and 0·096 grm. per day at the end of the experiment. Pups 2 and 4 had 4 grm. butter in place of 10 grm. of bread.

Slight enlargement of the wrists was observed in No. 1 on November 3rd and the pups were X-rayed, but the photographs showed no marked changes.

On November 22nd the dogs were put in pairs in metabolism cages and the urine and fæces collected for three days.

On November 29th another metabolic experiment extending over three days was made.

The details of these experiments (XVa and XVb) and of a similar metabolic experiment in Experiment XVIII (p. 183) are to be published by Dr. Telfer elsewhere. The main results are shown in Table VII.

On December 3rd X-ray photographs were taken of the fore limbs and showed very slight signs of rickets in pup 1 and pup 4, and no rickets in pup 2 and pup 3, and on December 19th distinct rachitic changes were manifest in pup 1 and pup 4, but no change in pup 2 and pup 3 (Plate I, figs. 3 and 4).

On December 19th pup 2 died under chloroform, and blood was collected from the heart for examination. On December 22nd pups 1, 3 and 4 were killed at the age of 20 weeks. It was noted that the bones of pup 1 were softer than the others.

The examination of the bones gave the following results.

TABLE IV.—*Analysis of Bones (Femora) (weights in grms.).*

Dog.	Weight of dogs.	Weight of femora.	CaO grms. per kilo dog.	Per cent.	P_2O_5 grms. per kilo dog.	Per cent.
Calcium-rich Nos. 2 and 3.	9070	10·4	0·58	24·55	0·46	17·8
Calcium-poor Nos. 1 and 4.	9000	8·9	0·49	22·3	0·40	15·6

It will be seen that the dogs on the calcium-rich diet had appreciably more mineral matter in their skeletons than those on the calcium-poor diet.

TABLE V.—*CaO Content of Muscle (in mg. per 100).*

	CaO.
Pup 2 (calcium-rich)	0·0145
Pup 3 ,,	0·018
Pup 1 (calcium-poor)	0·015
Pup 4 ,,	0·017

There was no appreciable difference in the calcium of the blood of the two pairs of animals.

Experiment XVIII.—This experiment was a repetition of the last, with the intake of calcium increased in two dogs to about eight times the intake of the other two, in the absence of any source of vitamin A other than lean meat and of direct sunlight. The metabolism of calcium and of phosphorus and the gain of each in the skeleton were studied and the distribution of calcium and of phosphoric acid in the blood was investigated.

Four pups, aged 4 weeks, of the same litter were put up on May 2nd, 1922, in pens in a room with a concrete floor into which direct sunlight never fell.

TABLE VI.—*Diet per Dog per day in grms.*

	2nd May to—	15th May to—	22nd May to 11th July.
Bread (dry)	78·5	105	105
Beef (lean)	50	75	77·5

This diet yielded on an average per kilo per day—

CaO	0·03	0·03	0·024	0·016
Fat	1·8	1·8	1·5	1·0
Calories	154	147	117	76

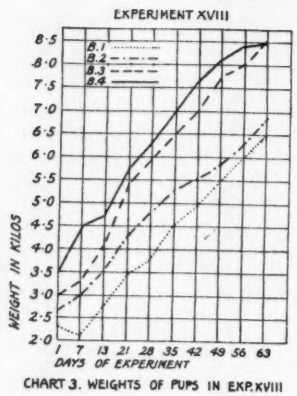

CHART 3. WEIGHTS OF PUPS IN EXP. XVIII

Pups Nos. 1 and 3 had, in addition, 4 grm. calcium lactate=0·73 grm. CaO, giving about 0·28 grm. per kilo, as against 0·03 to 0·016 grm. per kilo in Nos. 2 and 4.

The growth curves are shown in Chart. 3.

Between June 15th and 20th pups 2 and 4 showed weakness of the legs, and on June 21st pup 4 developed tremors resembling tetany, but the electrical reactions were taken and found to be normal.

On June 30th a metabolic experiment of four days' duration was conducted on pups 3 and 4, with the results shown in Table VII. (The details of the experiment are to be published by Telfer.)

TABLE VII.—*Intake and Retention of Calcium as CaO in the three Metabolic Experiments from Experiments XV and XVIII (calculated in grms. per kilo of body-weight per day).*

	CaO.					
	Calcium-rich (1 and 3).			Calcium-poor (2 and 4).		
	XV(a)	XV(b)	XVIII	XV(a)	XV(b)	XVIII
Intake	0·198	0·16	0·148	0·02	0·02	0·02
Retention (actual)	0·153	0·127	0·14	0·016	0·016	0·02
Retention (per cent. of intake)	77	79	94	80	80	100
	P_2O_5					
Intake		0·12	0·17		0·12	0·16
Retention (actual)		0·086	0·13		0·005	0·056
Retention (per cent. of intake)		72	76		4*	37

On June 22nd X-ray photographs taken showed pups 1 and 3 to be normal, pups 2 and 4 to have signs of rickets (Plate I, figs. 5 and 6).

On July 7th pups 3 and 4 were killed, and on July 11th pups 1 and 2 were killed, age a little over 16 weeks. The bones of pups 2 and 4 were soft.

The longitudinal sections of the ends of the ulna of Nos. 1 and 2 are shown in Plate I, figs. 7 and 8).

TABLE VIII.—*Calcium and Phosphorus in Blood (in mgs. per 100 c.c.).*

	CaO.		P.	
	Whole blood.	Serum.	Inorganic of serum.	Total of whole blood.
Pup 1 (calcium-rich)	10·9	15·3	5·7	42·5
Pup 3 ,,	10·9	14·6	6·0	35·4
Pup 2 (calcium-poor)	11·6	15·2	4·7	27·0
Pup 4 ,,	10·1	14·8	4·8	46·7

* In dealing with such small quantities, the error in calculating a percentage is, of course, very large.

TABLE IX.—*The CaO Content of the Muscle per cent.*

No. 1	0·014	(calcium-rich)
No. 3	0·014	,,
No. 2	0·016	(calcium-poor)
No. 4	0·015	,,

The histological examination of the costo-chondral junction was made by Prof. Korenchevsky, who reports as follows:

No. 1. *Nearly normal.* Zone of prov. calc. normally impregnated with lime. Slight osteoporosis due to the increased number of osteoclasts. Osteoclasts in most places normal. Osteoid is present in physiological amount. Hyperæmia of bone-marrow.

No. 3. *Not very far from normal.* Slightly increased proliferous cartilage and curved line of costo-chondral junction. Very few small sinuses are sometimes present under the line of costo-chondral junction. In a few places amount of osteoid seems to be slightly increased. "Reticulum" bone-marrow in spongiosa.

No. 2. *Rickets from calcium starvation.* Proliferous cartilage greatly increased. Lime deposition in the zone of provisional calcification deficient. Line of costo-chondral junction disorganised, but without deep irregular ingrowth of blood-vessels into cartilage. Large sinuses with thin walls often are present just beneath the line of costo-chondral junction. Trabeculæ of spongiosæ are interlaced and curved, consisting of osteoid tissue with more or less calcified central part. The largest amount of osteoid is equal to or sometimes exceeds the calcified part in the trabeculæ near the line of costo-chrondral junction. In the trabeculæ further from the line of costo-chrondral junction osteoid is present in small amounts. A considerable amount of osteoid is sometimes found under periosteum. Usually there are normal amounts of osteoclasts, but in some places their number is increased. "Reticulum" bone-marrow in spongiosa near the line of costo-chrondral junction. Cartilage canals are also increased in their number.

No. 4. *Rickets.* Picture similar to that of puppy No. 2, but less severe. Proliferous cartilage greatly increased. Sinuses beneath the line of costo-chondral junction. There is no irregular ingrowth of blood-vessels into cartilage. Lime is deposited only in a few places of zone of provisional calcification. Many trabeculæ of primary spongiosa, consisting of osteoid, imperfectly impregnated with lime. In general, osteoid all through, increased, and present in small amount. Osteoporosis.

An examination of the limb bones showed that in the dogs on the calcium-excess diet they were harder than were those in the dogs on the calcium-poor diet. When split they showed well-marked differences in the cortical thicknesses of the shafts. Those from the animals on the calcium-excess (No. 1 and No. 3) were rigid and quite obviously heavier and stronger than the bones of No. 2 and No. 4 (calcium-poor diet), which could easily be bent and twisted. These naked-eye differences of the amount of mineral matter contained in the contrasted specimens were corroborated by the analyses, shown in the following table:

TABLE X.—*Analyses of Bones* (*Femora*) (*weights in grms.*).

Number.	Weight of dog.	Femur. Weight per kilo.	CaO. Per kilo of dog.	CaO. Per cent.	P_2O_5. Per kilo of dog.	P_2O_5. Per cent.
Calcium-rich						
1	6000	1·876	0·425	22·6	0·328	17·5
3	8600	1·811	0·343	18·9	0·259	14·9
Calcium-poor						
2	5900	1·808	0·269	14·9	0·213	11·8
4	8450	1·197	0·158	13·2	0·13	10·8

The ratios $CaO : P_2O_5$ in the bone ash were practically identical in the two series. An excess of calcium in the diet led to an increased fixation of calcium in the skeleton. At the same time a corresponding increased quantity of phosphoric acid was fixed by this calcium, as indicated by the retention results in the metabolic experiments. In all these experiments the amounts of phosphoric acid in the various diets were identical. For conclusions from these experiments see p. 193.

(2) *Olive Oil in the Production of Rickets. Is its Action due to the Absence of a Vitamin?*

Experiment XVII.—The regularity with which all the pups treated by Mellanby with olive oil developed rickets raises the question whether a mere absence of some vitamin is the determining factor or whether olive oil may act in some other way.

To test this, on April 5th a set of four pups at 4 weeks old was used, with four others as checks. We were fortunate in having at the same time 16 pups of two litters of practically the same age, which were used for this and the following investigations.

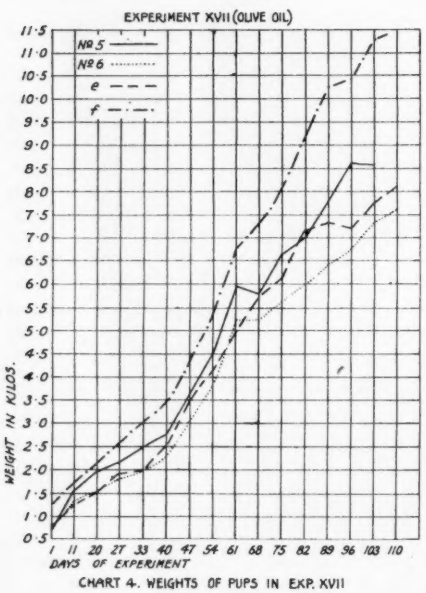

CHART 4. WEIGHTS OF PUPS IN EXP. XVII

Those of the first litter, A, are designated by numbers, those of the second litter, B, by small letters.

The basal diet in all consisted of dried separated milk and dried bread in the proportion of from 22 to 35 grm. of the former and 30 to 40 grm. of the latter, and the calories from 200 to 300 per kilo.

The diets were increased in seven stages as the dogs grew, in order to keep the food supply per kilo as nearly equal as possible. Immediately before an increase was made on account of growth, the energy value of the diet naturally fell below the mean, and immediately after the increase was made the mean of the diet was slightly exceeded.

In four of the pups 10 grm. of the bread were replaced by 5 c.c. of olive oil, keeping the energy value of the diet the same, and after five weeks the oil was increased to 10 c.c. The growth curves are shown in Chart 4.

After 20 days two of the four (Nos. 5 and 6) showed splaying of feet, and X-ray examination indicated ricket-like changes (Plate II, figs. 9 and 11).

A fortnight later No. 6 got 10 c.c. cod-liver oil in place of olive oil and after another fortnight the animal appeared to be better. Nos. 5 and 6 were

again X-rayed (Plate II, figs. 10 and 12). A marked improvement was observed in No. 6, but in No. 5 the rachitic process had distinctly advanced at the end of the radius. The pups were killed at the age of 19 weeks, after 14 weeks upon the diet. The general result of the experiment is shown below:

Checks.		Olive oil.
1 normal	3 normal	5 rickets
2 „	4 „	6 „
a „	c „	e normal
b „	d „	f „

In cutting longitudinally the lower ends of the radius and ulna, the appearance under X-ray was confirmed; there was marked ingrowth of bone into the epiphyseal cartilage in No. 6, but none in No. 5. The bones of No. 5 were not so hard as those of No. 6 (Plate II, figs. 13 and 15).

TABLE XI.—*Analysis of the Bones.*

Number.	Weight of dog.	Weight of femur.	CaO grams.	CaO per cent. of dog femur.
1	7000	16·41	4·06	24·7%
5	8500	17·15	3·79	22·1%
6	7600	15·35	3·92	25·5%

Prof. Korenchevsky kindly undertook the histological examination of the costo-chondral junctions and I am indebted to him for the following report:

No. 1. Nearly normal.

No. 5. ? Early rickets, increased proliferation of cartilage, osteoid slightly increased, slight osteoporosis, "reticulum" bone marrow.

No. 6. Nearly normal.

TABLE XII.—*Calcium Oxide and Phosphorus in Blood in mg. per 100 c.c.*

Number.	CaO. Whole blood.	CaO. Serum.	P. Inorganic of serum.
1. Check	11·74	—	4·9
5. Olive oil (rickets)	11·4	14·8	4·7
6. Olive oil, then cod-liver oil	11·6	14·5	6·2
8. Olive oil (no rickets)	9·4	13·7	5·5

In this experiment the addition of olive oil to a diet of 4 pups upon which 12 pups were reared under exactly the same conditions, free of any signs of rickets, induced the onset of the condition in two out of four animals. The two animals affected were of the same litter.

Manifestly the result cannot be attributed to lack of vitamin in the oil, nor can it be ascribed to a disturbance in the proportions of fats to carbohydrates. On the olive-oil diet these approximate closely to the proportion in

which they would occur if whole milk had taken the place of separated milk in the basal diet. Calculated from the basis of 10 grm. of protein, the proportions are :

TABLE XIII.

	Protein (grms.).	Fats (grms.).	Carbohydrates (grms.).
Separated milk diet	10	0·5	28·8
Olive oil diet	10	4·6	24·5
Whole milk diet	10	4·8	31·5

The X-ray pictures and the section of the ulna of No. 5 (Plate II, figs. 9, 10 and 13) further show, in a striking manner, what is well known in children—that the condition may advance in one bone or set of bones and not in others.

A further investigation of the action of various fats upon the absorption and fixation of calcium and phosphorus is urgently required. The older work of Rothberg, Steinitz, Meyer and Orgler from 1903 to 1907 (quoted by Findlay) seems to show that while milk fats have a prejudicial effect on the retention of calcium, cod-liver oil has a beneficial influence.

As already indicated, the more recent work of the American investigators —Hess, McCollum and his co-workers—has fully confirmed these observations, and has shown that the active substance in cod-liver oil, unlike vitamin A, resists prolonged heating and oxidation, so that the oil retains its property of increasing the fixation of calcium after it has lost its protective action against xerophthalmia.

A hint as to the possible way in which olive oil might interfere with the absorption of lime is afforded by the work of Pavloff's school and by the recent work of Lockwood and Chamberlain (1922), which shows a decrease in gastric activity and delay in emptying the stomach when olive oil is taken. This action may increase the alkalinity of the intestinal contents and may possibly interfere with the absorption of lime.

The results of the metabolic experiments (Table VII) showing the very complete absorption of calcium on a calcium-low diet renders an explanation of the mode of action of cod-liver oil and of light extremely difficult.

The absence of any change in the proportion of CaO or of the inorganic phosphates of the blood is clearly shown. The fact that in this and in the succeeding experiment the pups affected were from the same litter emphasises the necessity of using animals of the same litter in such investigations.

(3) *The Influence of Subdued Light.*

Experiment XIX.—We have shown that pups may be reared free of rickets on a diet of bread and separated milk, when kept in a well-ventilated room in the laboratory, exposed to sunlight through an ordinary glass window which filters off the rays which are said to be effective.

It seemed of interest to ascertain if further exclusion from light would produce any effect.

Four pups, aged 4 weeks, were used for this experiment, while the same eight that were used as checks in Experiment XVII were also used in this. All were from the two litters. The pups in one litter are indicated by numbers and those from the other litter by letters.

Checks.				Subdued light.
1	.	3	. . . .	7
2	.	4	. . . .	8
a	.	c	. . . .	g
b	.	d	. . . .	9

When put in a subdued light, aged 4 weeks, the pups at once became very lethargic, and, instead of constantly running about in their coop, fighting and barking, as did the pups in sunlight behind glass, they spent most of their time sleeping in a corner. At first they lost appetite, and for 5 weeks did not take all their food.

The growth curves are shown in Chart 5.

After 3 weeks it was noticed that 7 and 8 had enlarged knees, and that g was splay-footed. X-ray photographs at this date were taken (Plate I, fig. 1), and showed—

7 rickets. g normal.
8 ,, 9 ,,

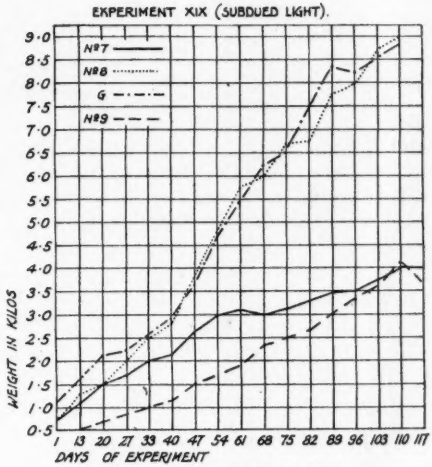

CHART 5. WEIGHTS OF PUPS IN EXP. XIX.

Nos. 7 and 8 were then given 10 c.c. cod-liver oil daily. After 4 weeks their condition was manifestly improved and X-ray examination showed the bones to be normal (Plate I, fig. 2).

The pups were killed on July 17th, at 22 weeks of age. The bones were all hard. Analysis gave the following results:

TABLE XIV.

Number.	Weight of dog.	Weight of femur.	CaO. Grams.	CaO. Per cent.
8	9000	9·36	2·21	23·6
9	4000	7·75	1·94	25·0

TABLE XV.—*The CaO and P of the Blood in Mg. per* 100 *c.c.*

	CaO. Whole blood.	CaO. Serum.	P. Inorganic in serum.	P. Total P in whole blood.
Check	11·7	—	4·9	36·3
Subdued light (rickets)	11·33	14·2	5·5	30·2
Subdued light (normal)	10·1	15·5	5·5	—

One point of no little interest is that while No. 8 showed not only healing of the rachitic process, but also a steady gain in weight, No. 7 showed healing, but little or no gain in weight, while the hair fell out and the coat became thin. Had this dog been alone under treatment, it might have been urged that the improvement was due to the stoppage of growth—an explanation which has been used to explain the improvement in rickets during starvation in rats (McCollum, 1922). But the fact that the same improvement occurred in its brother pup which increased in weight shows that this conclusion is unwarranted.

In this, as in the olive-oil experiment, it was the pups of litter A which developed rickets; that from litter B remained normal, as did its brother and sister in the olive-oil experiment. No more convincing proof could be found of the fact that certain breeds of dogs are more susceptible to rickets than are others, and that it is absolutely necessary in all experiments to compare pups of the same litter.

The experiment seems to indicate that one effect of exclusion from light is to bring about a lethargic condition in pups, and this may play its part in favouring the onset of the disease. From our previous experience on the effects of open-air life in accelerating growth of pups, we are inclined to agree with Powers, Park, and Simmonds (1922) (p. 177).

(4) *The Influence of Infection.*

The association of rickets with unhygienic surroundings and its familial incidence, as well as its mode of onset, progress and recovery, have suggested the possibility of its being primarily of microbial origin. It is not necessary to assume that the infection is a specific one. It might be of the nature of the infection of water which McCarrison has shown to be associated with the development of goitre. It is even possible that a change in the intestinal flora might alter the reaction of the intestinal contents towards the alkaline side and might thus limit the absorption of lime and phosphoric acid.

In our experience, if pups are kept healthy and free from diarrhœa and vermin, they do not develop ricket-like changes in the bones, unless the intake of calcium is markedly cut down. On the other hand, they may develop rickets on an ample intake of calcium and vitamin A in milk, if not kept clean (Renton and Robertson, 1916–17).

Moussu (1903) states that a healthy dog developed rickets when kept in a cage with a rachitic one, and that a second healthy dog, put in the same cage without disinfection, developed the disease. Bull's account (1918) of an outbreak of rickets in fox-hounds strongly suggests an infective element in the onset of the disease.

The evidence of previous investigators has taken the direction of trying to find micro-organisms in the epiphysial region of the bones or of various organs. Such methods do not seem likely to throw much light upon the question and the results are inconclusive. Morpurgo (1900) alone has isolated a diplococcus from the marrow of rats affected with spontaneous rickets, and by injecting it into other rats produced bone changes resembling those of rickets.

In considering how the infective process might be established in the child, it seemed that it might occur either through the blood, by transmission through fleas, lice, or bugs, or by infection through the alimentary tract. The latter view was supported by Marfan (1911). Iovane and Forte (1907) state that they produced rickets in rabbits by injecting extracts of the fæces of rickety children.

An attempt has been made to investigate these possibilities, first, by injecting the blood of rachitic children into pups, and, second, by adding to the food of pups the fæces of children suffering from active rickets.

It is, of course, evident that in such an experiment a positive result would be of great significance, but a negative result would not prove the non-infective nature of rickets.

It may well be that before an infection can be effective, the vitality of the tissues must be lowered by some preceding illness or other condition, and it is also possible that infection from man to dog may not be transmissible, and that canine may not be identical with human rickets.

The following experiments have been performed:

Experiment XIII.—The object of this experiment was to test whether rickets might be produced in pups by the injection of blood from cases of active rickets.

A litter of five pups was put up in two lots at the age of four weeks. In order to obviate the possible criticism, that if rickets developed in any, it was due to a diet with an insufficient supply of vitamin A, the diet was as follows:

TABLE XVI.

	June 15th (grms.).	August 24th (grms.).
Full cream milk (dried) (Cow & Gate)	45	56
Bread (dried)	60	75
Containing:—		
CaO	0·588	0·73
Fat	10·176	12·705
Calories	469	585

The caloric value was, on an average, about 180 to 156, and 135 at the later date, per kilo of body-weight.

From June 22nd pups 1, 2 and 3 got 1 c.c. of blood subcutaneously once a week till July 28th, when No. 2 died, without any signs of rickets, apparently from some interference with deglutition.

By mistake, during August, No. 4 was injected instead of No. 3. Thus all had injection of blood except No. 5.

On September 26th—after twelve weeks—the dogs were all healthy and well-grown and none showed any signs of rickets.

Experiment XII.—This experiment was carried out to find if rickets could be induced in pups by the administration of fæces from cases of active rickets.

A litter of five pups aged four weeks was put up in two lots on May 5th, 1921. The diet selected was—

TABLE XVII.

	May 5th (grms.).	June 29th (grms.).
Full cream milk (dried) (Cow & Gate)	22·5	34
Bread (dried)	30·0	50
Containing :—		
CaO	0·294	0·448
Fat	5·083	7·745
Calories	235	373

The calories per kilo were, on an average, 140 on May 5th and 132 on June 29th.

To the food of Nos. 1, 2 and 3 about 3 grm. of fæces from cases of active rickets were added. Nos. 4 and 5 were used as checks.

X-ray examination on July 12th showed no rachitic change.

They were killed on July 27th.

The bones of Nos. 4 and 5 were softer than those of Nos. 1, 2 and 3, but no signs of rickets were observed.

Dr. Coulthard, of the Pathological Department, kindly made a series of bacteriological examinations of the fæces, and he reports :

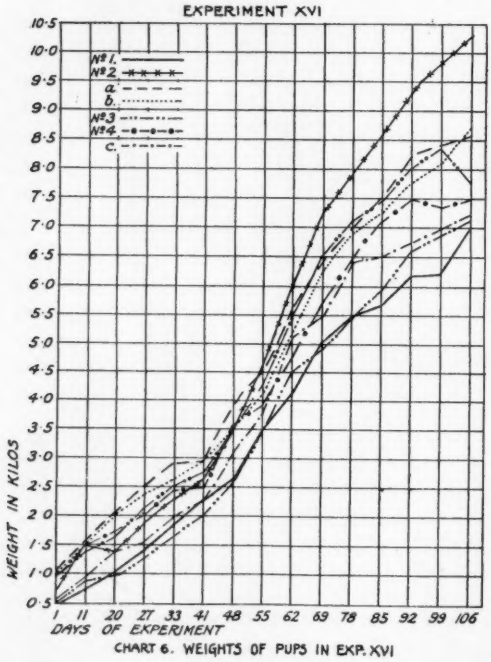

CHART 6. WEIGHTS OF PUPS IN EXP. XVI

"In general, the flora throughout the series showed no great variation, coliform bacilli, streptococci, Gram-positive bacilli of anaërobic types being abundant in all. *B. acidophilus* was present in all, but was most notably abundant in No. 1. Coliform bacilli, as shown by cultures on McConkey's medium, were abundant in all, and when tested for fermentation reactions, etc., were found to be of the common varieties (where there were distinct differences in the appearances of colonies, several were always tested from the same animal). On only one occasion (No. 1) was a non-lactose fermenting bacillus met with; it formed indol, but did not liquefy gelatine, and was somewhat similar to Morgan's bacillus. *Anaërobes.*—There were no striking variations in character or numbers in the series."

Experiment XVI.—This is a repetition of XII, but the vitamin content of the diet was reduced by substituting separated dried milk for full-cream dried milk.

Eight pups from two litters, as described on p. 186, were used, four serving as checks and four getting fæces in their food.

The diet is described on p. 186. All the pups grew well (Chart 6), were lively, and none showed any signs of rickets. The experiment terminated on July 11th, 1922, the pups being then 19 weeks old.

The result of the examination showed that both series were normal.

This series served as checks to the experiments upon olive oil and subdued light (pp. 186–188).

Further experiments upon the possible infective nature of rickets in rats are now in progress.

These experiments do not support the infective theory, but it is very possible that dogs may be resistant to infection from the human subject. The experiments should be repeated with material derived from rachitic dogs.

CONCLUSIONS.

(1) A limitation of the supply of calcium proved to be of more importance in determining the onset of rickets than the limitation of vitamin A, since all other factors except the intake of calcium were the same in the normal and in pups showing ricket-like changes (pp. 180–186).

(2) A very restricted supply of calcium, although leading to changes in the bones, chemically and structurally, resembling those found in rickets, did not materially reduce the calcium of the blood and muscles, or the inorganic phosphorus content of the blood.

(3) The retention of phosphorus appeared to be determined by the retention of the calcium (p. 184).

(4) The percentage of the calcium retained did not vary very markedly with the amount in the food, within the limits investigated of 0·02 to 0·198 grm. CaO per kilo. The very high percentage retention of lime in the calcium-poor diet makes it difficult to explain the favourable action of cod-liver oil upon the retention (p. 184).

(5) A comparison of the condition of pups exposed to sunlight through glass with those from which direct sunlight was excluded showed that the latter condition favoured the onset of rickets, but whether the result was due to absence of the direct action of sunlight or whether it was associated with the lethargy manifested by these pups is not indicated. In this connection, the remarks of Park and his colleagues, given on p. 178, are worthy of attention (p. 188).

(6) The addition of olive oil to a diet which was adequate to prevent rickets actually favoured its onset. This can hardly be explained by deficiency of the oil in some antirachitic substance (p. 186).

(7) Cod-liver oil exercised a marked effect in favouring the cure of rickets in pups (pp. 187, 189).

(8) The addition of fæces from cases of infantile rickets to the diets of pups, whether upon a diet of full cream, milk and bread, or upon a diet of separated milk and bread, was not followed by a development of rickets (p. 191 *et seq.*).

(9) The injection of blood from cases of active infantile rickets did not produce the condition in pups upon a diet of full cream milk and bread (p. 191).

While these experiments give no support to the theory that rickets is an infective disease, they, of course, do not disprove the possibility that a particular condition of the intestinal flora, which might be propagated by fæcal infection, may so modify the absorption of calcium and phosphorus as to lead to rachitic changes.

(10) These experiments further show very clearly that, in testing the influence of any factor in producing rickets, it is necessary (*a*) to use sets of animals and not single animals, and (*b*) to use pups of the same litter.

It has been repeatedly shown that in rats the limitation of the supply of calcium and phosphorus to the bone may produce ricket-like changes, but how the limitation is brought about in infants has never been demonstrated. While we have previously argued against the probability of decreased absorption being the cause of failure in ossification, the work of Telfer and others has shown that decreased absorption cannot be excluded as a factor, and that further study of the intestinal conditions during the development of rickets is required.

We desire to thank Dr. Telfer and Dr. Grace Anderson for the analyses of the calcium and phosphorus of the bones, blood and muscles, which they were good enough to carry out for us.

The expenses of the investigation were defrayed by the Medical Research Council, to which our thanks are due.

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Paton and Watson. Plate II.

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EXPLANATION OF PLATES.

PLATE I.

FIG. 1.—X-ray photograph of the ends of the radius and ulna from pup 7, Exp. XIX, after 3 weeks in subdued light.

FIG. 2.—The same, after 7 weeks on cod-liver oil, showing cure.

FIG. 3.—X-ray photograph of the ends of the radius and ulna from pup 2, Exp. XV, after 9 weeks on calcium-rich diet.

FIG. 4.—X-ray photograph of the ends of the radius and ulna from pup 4, Exp. XV, after 9 weeks on calcium-poor diet.

FIG. 5.—X-ray photograph of the ends of the radius and ulna from pup 2, Exp. XVIII, after 5 weeks on calcium-poor diet.

FIG. 6.—X-ray photograph of the ends of the radius and ulna from pup 3, Exp. XVIII, after 5 weeks on calcium-rich diet.

FIG. 7.—Photograph of longitudinal section of the end of the ulna of pup 1, Exp. XVIII, on calcium-rich diet.

FIG. 8.—Photograph of longitudinal section of the end of the ulna of pup 2, Exp. XVIII, on calcium-poor diet.

PLATE II.

FIG. 9.—X-ray photographs of the end of the radius and ulna from pup 5, Exp. XVII, after 7 weeks on olive oil.

FIG. 10.—The same after a further 7 weeks on olive oil.

FIG. 11.—X-ray photographs of the end of the radius and ulna from pup 6, Exp. XVII, after 7 weeks on olive oil.

FIG. 12.—The same after 7 weeks on cod-liver oil.

FIG. 13.—Photograph of longitudinal section of the end of the ulna of pup 5, Exp. XVII, on olive oil.

FIG. 14.—The same of pup 8, Exp. XVII, on olive oil, but no rickets.

FIG. 15.—The same of pup 6, Exp. XVII, on olive oil, followed by cod-liver oil.

EXPERIMENTS ON THE RESPIRATION OF MALIGNANT TISSUES.

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Received for publication April 24th, 1923.

THERE is one aspect of the metabolism of tumours which has as yet been investigated very little, viz. the respiration. Although the literature upon the subject of nitrogenous metabolism and proteolytic enzymes is exceedingly extensive and but little perspicuous, the works upon oxidation are few enough to permit a review within a limited space.

In Blumenthal's great compilation, 'Die chemischen Vorgänge bei der Krebskrankheit,' published in 1910, there is not a single line on the subject of respiratory exchange, while Herxheimer and Reinke, in their monograph upon the 'Pathologie des Krebses,' which appeared three years later, could cite only one work on this subject, viz. one by Chisolm (1910), who studied the respiratory exchange of normal and cancerous mice without detecting any difference whatever. Two years before this Cramer (1908) had come to the same result in experimenting with rats.

It is only within the last few years that the respiration of tumour tissues has been studied. Most of this work has been done in the laboratories of the Imperial Cancer Research Fund, London, which has published the four English papers cited below.

Drew (1920) has compared the reducing powers of normal tissues on methylene blue with that of the neoplastic ones, and has come to the conclusion that the oxygen avidity of the tumours is considerably lower than that of normal tissue (kidney, liver, spleen, testis, heart, brain, mamma, connective tissue, "embryo"). His method was very simple: a fine pulp of the tissue was suspended in methylene-blue solution under liquid paraffin at a constant temperature of 37° C.; every half-hour during 5–6 hours the height of the discoloured layer was measured. Drew used solutions of 0·001–0·002 per cent. strength.

This difference between normal and neoplastic tissue was not confirmed by Russell and Gye (1920), who suspended the emulsified tissue (mouse) in rabbit blood saturated with oxygen at 37·5° C., and observed the consumption of oxygen by employing Barcroft's blood-gas apparatus. With every reserve, these investigators draw from their experiments the conclusions that the rate of oxygen consumption rises with increasing speed of growth, and further, that more oxygen is used by tumours which are more highly differentiated histologically.

Russell and Woglom (1920) measured the gaseous exchange of tumours and normal tissue (mouse) by employing Barcroft-Krogh's micro-respirometer. They do not venture upon any conclusions as to the respiration intensity, but they consider that the respiratory quotient of the tumours is proportional to the speed of growth.

Quite contrary to Drew's result, Waterman and Dirken (1921) found a higher oxygen consumption in tumour tissue (*homo*, chicken) than in normal tissue of comparable cellular density (kidney, heart, muscles). They suspended a fine emulsion of the tissue together with red blood-corpuscles in a Ringer solution, which then was saturated with oxygen; at given intervals the oxygen tension was estimated by Barcroft's method.

According to Brahn (1920), cancerous tissue as contrasted with normal tissue possesses no oxidising powers on salicylic aldehyde.

Quite recently Russell (1922) has tested the influence of various carbohydrates on the gaseous exchange of liver, kidney and tumour (mouse) without being able to detect any differences between the various tissues.

An account will be given here of some experiments, thus far preliminary, upon oxidation in human tumour tissue, obtained from the Surgical Clinic of the University, Lund. The oxidation is studied by employing Thunberg's methylene-blue method. As to the arrangement of the experiments I refer to my work, "Über die spontanreduzierende Einwirkung der Muskulatur auf Methylenblau" (Ahlgren, 1921).

The use of the methylene-blue method for studying biological oxidations depends upon the fact that methylene blue is a delicate indicator for the hydrogen which is liberated (activated) from oxidisable substances through the action of the dehydrogenases (Thunberg). Methylene blue as hydrogen acceptor thus plays the same *rôle* as does oxygen in the physiological processes of the tissues. For the theoretical basis of the methylene-blue method, *vide* Wieland (1922) and Thunberg (1917, 1920).

The fitness of the methylene-blue method for the determination of the absolute value of the respiration intensity of a tissue might reasonably be questioned, for its action is not limited to that of a passive hydrogen acceptor, but apparently we have also to do with a catalysis (Meyerhof, 1917, 1918; Thunberg, 1922; Wishart, 1923). But for the solution of the questions treated in this report, as well as for the investigation of many problems concerning the mechanism of respiration, the methylene-blue method has indisputable advantages over gas analysis.

The m-dinitrobenzol method of Lipschitz (1920, 1921), shares with the methylene-blue method the above-mentioned disadvantage (Thunberg, 1922). From the point of view of Wieland's oxidation theory the double nature of m-dinitrobenzol as hydrogen acceptor and oxygen donator should render it a less suitable indicator of oxidation than methylene blue. Moreover, Lipschitz' method cannot be made so sensitive as the methylene blue method and its technique is more complicated. Furthermore, Waterman and Kalff (1923) have recently criticised it severely.

As regards the respiration intensity of tumour tissue, the experiments have proved the extremely great influence of the hydrogen ion concentration and of the presence of an adequate quantity of combustible matter in the tissue.

These factors have been quite overlooked or only insufficiently taken into consideration by previous investigators in this field—a circumstance which lessens the value of their results considerably, as well as explains the contradiction between them. Consideration of these factors is, of course, just as necessary when estimating the respiration intensity of the control tissue.

Very likely the respiratory quotient is also influenced by the factors mentioned.

Another important factor is the degree of mincing of the tissue, concerning which I refer to my report upon oxidation in the muscles, which will shortly be published (Ahlgren, 1923).

It has long keen known that in mincing various animal tissues acid products are formed which are capable of changing the hydrogen ion concentration more or less, a change which decidedly affects the action of the respiratory enzymes. The following experiment illustrates the necessity of regulating the hydrogen ion concentration by means of a sufficiently powerful buffer substance.

EXPERIMENT 1, 28 . vii . 1921: *Cancer simplex mammæ* from a 46-year-old woman. Mammary amputation under ether anæsthesia at 9.45 a.m. Soft, medullary, non-necrotic tumour of the size of an orange. A piece of the tumour was freed, as far as possible, from fat and connective tissue, and was minced with a pair of curved scissors.

Vacuum tube	1	2
Methylene blue 1 : 2500	0·25 c.c.	0·25 c.c.
m/10 potassium phosphate	—	0·40 c.c.
Distilled water	0·75 c.c.	0·35 c.c.
Tumour mass	0·30 gr.	0·30 gr.
In thermostat, 37° at	11.40 a.m.	11.46 a.m.
Decoloration time	50 min.	7·5 min.

In two other tubes similar mixtures were placed, but for the methylene-blue solution distilled water was substituted. When brom-thymol blue was added to these tubes, the pH was found in the first one to be below 6·2, in the second one about 7·0.

Probably the accelerating influence of the potassium phosphate on the oxidation may not be exclusively ascribed to its buffer action. The presence of potassium phosphate in a certain concentration seems to be necessary for the normal progress of the cellular respiration (Thunberg, 1909). The normal concentration of the potassium phosphate is, of course, lessened by suspension in methylene-blue solution.

Partly because of its character as a normal cellular constituent and partly because, in contra-distinction to several other buffer substances (*e. g.* citrate, acetate, glycocoll), it cannot be supposed to act as hydrogen donator, potassium phosphate is very suitably employed as buffer substance.

It has long been known that it is necessary to consider the temperature when testing the reaction velocities in physiology. But it is only in recent years that the importance of the hydrogen ion concentration to the enzymatic reactions—for instance to the oxidation in the tissues—has been perceived. Consequently when stating the respiration intensity of a certain tissue one must also tell the temperature and the hydrogen ion concentration at which it was estimated. As regards the temperature, it is natural to use that of

the body when working with warm-blooded animals. As to the hydrogen ion concentration, we meet with difficulties as we do not know the pH in the tissues. To use the hydrogen ion concentration at which the action of the enzymes is optimal (for the succino-dehydrogenase about pH = 8·7, according to Ohlsson, 1921) would be unsuitable, as such an alkaline reaction in a living tissue would hardly be conceivable. We find a perfect parallel to this in the temperature, as the most favourable level of temperature for certain dehydrogenases is above that endurable to the organism. For the present it will be most natural to estimate the respiration intensity at a hydrogen ion concentration like that of the blood.

It is astonishing how little the investigators, who have worked upon the respiration of isolated animal tissues, have taken into consideration the error due to the differing percentage of combustible matter. By means of one method or another, one has estimated the gaseous exchange, and a tissue which shows a higher value is simply supposed to have a greater respiration intensity than one that shows a lower value. The fact is overlooked that the lower value may be due to the possibility that the tissue in question has such a great respiration intensity, that the available quantity of combustible matter has been consumed after the separation of the tissue from the system, just as a machine slows down when the supply of fuel is lessened. As is pointed out by Russell, who, in his last work (1922), treats of this source of error, it is well to estimate the gaseous exchange as soon as possible after removing the tissue from the mother organism. But even so, one never knows what influence the source of error has had. Moreover, lack of combustible matter does not necessarily ensue upon the removal of the tissue from the organism. Quite specially in the case of malignant tumours, and perhaps also of certain of the benign ones, one is entitled to look upon such a lack as being possible even *in situ* in the organism; the spontaneous necrotic decomposition which is here quite frequent might of course be due to deficient supply of food-stuffs. In my work (1921) upon the reducing powers of the muscles on methylene blue I have shown that, in the muscles, the concentration of the substances to which the oxidation enzymes are accommodated very often falls below that at which the enzymes in question act optimally. In my continued investigations I have found that such is also the case in most organs of the body.

It should also be remembered that in the experiment itself a decrease takes place in the concentration of the combustible matter by the suspension of the tumour mass in a bigger or smaller quantity of liquid.

Of course, in order to estimate the oxidising powers of an isolated tissue, one must see to the presence of a sufficient quantity, not only of oxygen (or of some other hydrogen acceptor), but also of the substances that are to be oxidised.

The only way to get a conception of the extent to which the value found for the respiration exchange in a tissue is influenced by a larger or smaller supply of combustible matter, is to estimate the respiration exchange both with and without a surplus of this material added to the medium in which the oxidation takes place. The greater the difference in these two cases, the poorer is the tissue in oxidisable substances.

As to the choice of such available substances for these experiments which could serve as combustible matter in tissues, there are several possibilities.

Glucose and other carbohydrates prove to be unsuitable, as at least most pulped tissues have a very small capacity of consuming them—the amount of insulin present may perhaps here be important. It would be interesting in itself to add a mixture of all the various substances (aliphatic acids, amino acids, aldehydes, etc. (Ahlgren, 1923)), for the catabolism of which the tissues are provided with more or less specific chemical equipment, viz. enzymes of different kinds. Our insufficient knowledge of these substances and of their proper mixing proportions blocks this way. Another way would be to use extracts of fresh organs. Several investigators have applied this method in restituting a metabolism which has ceased because of the consumption of the substrate or because of its elimination by washing. In my work on oxidation in the muscles, mentioned above, I have advantageously used for this purpose an addition of potassium succinate, succinic acid being a substance very easily consumed by most of the organic tissues; it will also fill a very large place in the intermediary metabolism. It has proved suitable also in my experiments with tumours. In some experiments I have also used potassium glycerophosphate (in neutral solution); that, however, unlike the succinate, has the disadvantage of being a strong alkaline buffer substance. Occasional experiments with lactate, acetate, etc., have not succeeded very well.

Here follow the protocols of the experiments in which I have estimated the reducing powers of tumours upon methylene blue, partly without, partly with an addition of potassium succinate or potassium glycero-phosphate. In some of the experiments the reducing powers of muscles from the same patient have been estimated simultaneously—this was done when muscles were extirpated at the same time as the tumour, *e.g.* at mammary amputations. On account of my other duties, two hours at the least, often four or still more have passed between the extirpation of the tumour and the estimation of its reducing powers (transportation, preparations).

EXPERIMENT 2, 12 . i . 1920: *Cancer (gelatinosus) coli sigm.*—Intestinal resection under ether anæsthesia at about 10 a.m. At about 3 p.m. piece of tumour was excised, was washed cautiously in physiological saline, and pulped with a pair of curved scissors.

Vacuum tube		1	2
Methylene blue 1 : 2500		0·5 c.c. (200 γ)	0·5 c.c.
Distilled water		0·5 c.c.	0·5 c.c.
Potassium succinate		—	1 knife's point
Tumour mass		0·2 grm.	0·2 grm.
In thermostat 35° at		3.19 p.m.	3.19 p.m.
	at 3.24	—	+*
	3.39	(+)	++
	3.49	+	+++

EXPERIMENT 3, 5 . vii . 1921: *Sarcoma metastat. reg. lumb.* (round-cell sarcoma) from 59-year-old woman. Extirpation under local anæsthesia at

* +++ indicates complete decoloration; the other + signs various degrees of decoloration.

9 o'clock a.m. At 11.30 tumour was dissected free, necrotic parts were pared off, remains washed cautiously with physiological saline, slightly dried with filter-paper, pulped with a pair of curved scissors.

Vacuum tube	1	2
Methylene blue 1 : 5000	0·5 c.c. (100 γ)	0·5 c.c.
m/10 potassium succinate solution	—	0·2 c.c.
Distilled water	0·5 c.c.	0·3 c.c.
Tumour mass	0·2 grm.	0·2 grm.
In thermostat 35° at	12.2 p.m.	11.59 a.m.
at 12.30	—	++
12.55	—	++(+)
1.2	(+)	+++
2.50	++	—
3.20	+++	—
Decoloration time	202 min.	63 min.

EXPERIMENT 4, 21 . vii . 1921: *Cancer (simplex) mammæ* from 63-year-old woman. Mammary amputation under ether anæsthesia at 10 a.m. Hard tumour; was freed, as far as possible, from fat and connective tissue, was minced and triturated cautiously in a mortar into a fine pulp.

Vacuum tube	1	2
Methylene blue 1 : 2500	0·25 c.c. (100 γ)	0·25 c.c.
m/10 potassium phosphate solution	0·3 c.c.	0·3 c.c.
m/10 potassium succinate solution	—	0·2 c.c.
Distilled water	0·45 c.c.	0·25 c.c.
In thermostat 35° at	12.26 p.m.	12.38 p.m.
Decoloration time	24 min.	14 min.

EXPERIMENT 5, 23 . vii . 1921: *Cancer (simplex) mammæ* from 65-year-old woman. Mammary amputation under ether anæsthesia at 10.45 a.m. Preparation as in Experiment 4 immediately before the experiment.

Vacuum tube	1	2
Methylene blue 1 : 2500	0·25 c.c. (100 γ)	0·25 c.c.
m/10 potassium phosphate solution	0·30 c.c.	0·30 c.c.
m/50 potassium succinate solution	—	0·05 c.c.
Distilled water	0·45 c.c.	0·40 c.c.
Tumour mass	0·3 grm.	0·3 grm.
In thermostat 35° at	1.15 p.m.	1.19 p.m.
Decoloration time	33 min.	22 min.

EXPERIMENT 6, 18 . viii . 1921: *Cancer (simplex) mammæ* and of muscular tissue of pectoralis muscle from 36-year-old woman. Mammary amputation under ether anæsthesia at 10 a.m. Rather soft tumour. Preparation as in preceding experiment, only no bruising in mortar. Muscular tissue emulsified with a pair of curved scissors. 37° in thermostat. In each tube 0·4 c.c. of methylene blue 1 : 1000 = 400γ.

Vacuum tube .	1	2	3	4	5	6
m/10 potass. phosph. sol. .	—	0·4 c.c.	0·4 c.c.	—	0·4 c.c.	0·4 c.c.
m/10 potass. succinate sol.	—	—	0·2 c.c.	—	—	0·2 c.c.
Dist. water .	0·6 c.c.	0·2 c.c.	—	0·6 c.c.	0·2 c.c.	—
Tumour mass .	0·3 grm.	0·3 grm.	0·3 grm.	—	—	—
Muscle mass .	—	—	—	0·3 grm.	0·3 grm.	0·3 grm.
In thermostat at .	12.7 p.m.	12.10	12.13	12.26	12.29	12.31
Decoloration time	143 min.	68 min.	38 min.	6 min.	2·5 min.	3 min.

EXPERIMENT 7, 16 . xi . 1922: *Cancer* (*medullaris*) *mammæ* and of muscular tissue of pectoralis muscle.—Mammary amputation at about 11 o'clock a.m. Preparation as in preceding experiment. 37° in thermostat. 0·5 c.c. of methylene blue 1 : 2500 in each tube = 200γ.

Vacuum tube .	1	2	3	4	5	6	7	8
m/10 potass. phosph. sol.	—	0·2 c.c.	0·2 c.c.	0·2 c.c.	—	0·2 c.c.	0·2 c.c.	0·2 c.c.
m/10 potass. succinate sol . .	—	—	0·2 c.c.	—	—	—	0·2 c.c.	—
m/10 potass. glycero-phosph. sol.	—	—	—	0·2 c.c.	—	—	—	0·2 c.c.
Distilled water .	0·5 c.c.	0·3 c.c.	0·1 c.c.	0·1 c.c.	0·5 c.c.	0·3 c.c.	0·1 c.c.	0·1 c.c.
Tumour mass .	0·2 grm.	0·2 grm.	0·2 grm.	0·2 grm.	—	—	—	—
Muscle mass .	—	—	—	—	0·2 grm.	0·2 grm.	0·2 grm.	0·2 grm.
In thermostat at	2.20 p.m.	2.22	2.25	2.32	2.48	2.54	2.57	3.00
at 5.40	+ +	—	—	—	—	—	—	—
Decoloration time	—	111 min.	53 min.	12 min.	9·5 min.	6 min.	6 min.	6 min.

EXPERIMENT 8, 20 . xii . 1922: *Cancer* (*simplex*) *mammæ* and of muscular tissue of pectoralis from 65-year-old man. Mammary amputation about 24 hours before the experiment. Preparation as in preceding experiment. 37°. In each tube 0·5 c.c. of methylene blue 1 : 2500 = 200γ. Buffer substance: 5 c.c. of m/10 K_2HPO_4 solution + 1 c.c. of n/10 caustic potash, 0·3 c.c. of which in each tube.

Vacuum tube . .	1	2	3	4	5	6
m/10 potass. succinate sol. . .	—	0·2 c.c.	—	—	0·2 c.c.	—
m/10 potass. glycero-phosph. sol. .	—	—	0·2 c.c.	—	—	0·2 c.c.
Distilled water .	0·2 c.c.	—	—	0·2 c.c.	—	—
In thermostat .	2.31 p.m.	2.33	2.36	2.58	3.01	3.07
at 8 p.m.	+ +	—	—	—	—	—
Decoloration time .	>330 min.	96 min.	55·5 min.	42 min.	14 min.	18 min.

In these experiments the importance of the greatest attention to these factors becomes obvious. It is, however, impossible to draw any further conclusions concerning the respiration intensity of malignant tumours. One would almost be inclined to ascribe a lower respiration intensity to the tumour tissue than to the muscles. However, further experiments are needed in this field.

The great increase in methylene-blue reduction caused by the addition of the potassium glycero-phosphate is especially remarkable, although in experiment 7, where the capacity of the phosphate buffer was relatively small, the buffer action of the glycero-phosphate might also have had some influence. As the oxidation of succinic acid and glycero-phosphoric acid is brought about by different enzymes (Ahlgren, 1922), experiments with simultaneous addition of these two substances ought to be made. These acids differ from all other substances hitherto tested that can be oxidised in the tissues, among other things in the extent of their accelerating influence upon the combustion. Also other substances ought to be tested as to their influence upon the tumour tissue in this respect.

Experiment 8 shows that the tumours, after 24 hours of preservation, have lost most of their spontaneous reducing powers upon methylene blue, in which respect they are like liver, kidney, and many other organs (Morgan, Stewart and Hopkins, 1922).

SUMMARY.

When studying tumour respiration one has not hitherto sufficiently considered either the hydrogen ion concentration or the supply of combustible matter in the tissue the respiration of which is to be tested. The importance of these factors is now made evident. The hydrogen ion concentration should be maintained at the normal blood reaction by a sufficiently powerful buffer substance, preferably by potassium phosphate. To estimate the respiration intensity there must be a sufficient quantity present not only of oxygen (or of some other hydrogen acceptor), but also of a combustible matter, this being consumed without replacement when the tissue is removed from the organism. This is suitably done by adding succinic acid or glycero-phosphoric acid, or both of them simultaneously.

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THE RATE AND PROGRESS OF BACTERIOPHAGE ACTION.

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The bacteriophage used in these experiments was isolated from the urine of a woman suffering from bilateral pyelitis due to *B. coli*. It is lytic for the organism grown from the urine of the same patient and for some other strains of *B. coli* as well.

This bacteriophage, when incubated with a broth culture of the autogenous bacillus, produces all the typical appearances which have been described as characteristic of bacteriophage activity. Details of these appearances have been published already (Lepper, 1923).

The active principle has been increased by incubating the bacteriophage in broth with the autogenous culture and subsequent filtration through a Berkefeld filter. After the ninth passage a filtrate was obtained which, in a dilution of 1/2500, was able to reduce to zero an inoculum of 100,000 of the autogenous bacilli per 1 c.c. A heterologous sensitive bacillus required a dilution of 1/500 to destroy completely the same number of organisms.

This sample of bacteriophage was used to test the rate of action of the lytic principle.

Method of Experiment.

The bacteriophage was diluted with peptone-water to a volume of 4·9 c.c.; 0·1 c.c. of an emulsion of bacilli was then added with a dropping pipette, the final volume, 5 c.c., containing the required concentration of bacteriophage.

As soon as the organisms had been added a stop-watch was started, and the mixture was thoroughly shaken for the first half minute. Samples were removed at intervals and drops from a standardised dropping pipette delivered on the surface of a thin layer of set agar in a petri dish. Each drop had a volume of 0·02 c.c.

When the stop-watch showed that the time for plating had arrived, 5 c.c. of melted agar cooled to 42° C. was poured into the dish and thoroughly mixed with the drops of culture.

When this layer had solidified 10 c.c. of melted agar was poured over its surface. The plates were then incubated for 24 hours.

This method of plating was used by Penfold (1914) when studying bacterial lag, and has the great advantage of preventing surface colonies.

The number of drops of the mixture of bacteriophage and bacilli taken for each plating was increased proportionately with the time so as to keep the number of colonies per plate as uniform as possible.

The number of bacilli inoculated was such that all the colonies on each plate could be counted.

In each experiment a control tube containing 4·9 c.c. of peptone-water was inoculated with 0·1 c.c. of the bacillary emulsion and plates were made from it to determine the number of organisms present.

In the charts which illustrate the following experiments the numbers given refer to the colonies which developed per drop (0·02) of the mixtures of bacteriophage and bacilli.

EXPERIMENT I.—*To investigate the rate of action of a dilution of* 1/100 *of the bacteriophage of the heterologous culture.*

The number of organisms inoculated was 12,650 per drop ; the temperature was 21° C.

The results obtained are shown in Chart 1, compiled from figures detailed in Table I.

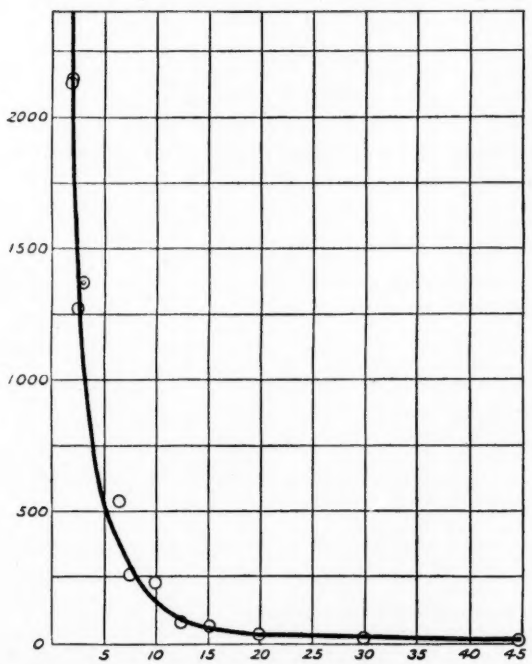

CHART 1.—The rate of action of bacteriophage on heterologous culture. Ordinates = number of bacteria surviving per drop after the action of bacteriophage for minutes (abscissæ).

TABLE I.

Time of plating.	Number of drops used.	Colonies on plate.	Average per drop.
0 min. 0 sec.	—	—	12,650
1 „ 40 „	1	2,134	2,134
1 „ 0 „	1	2,150	2,150
2 „ 30 „	2	2,544	1,277
3 „ 0 „	3	4,124	1,375
6 „ 30 „	6	3,267	544
7 „ 30 „	8	2,077	262
10 „ 0 „	10	2,283	228
12 „ 30 „	15	1,190	79
15 „ 15 „	20	1,353	67·6
20 „ 0 „	20	622	31
30 „ 30 „	30	460	15·3
45 „ 40 „	40	118	2·9

Discussion of Chart I.—The initial fall in the number of bacilli present is very rapid, the number of colonies per drop at the end of two minutes being only 2150 as compared with 12,650 in the control tube to which no bacteriophage had been added.

After the first five minutes the rate of fall is less rapid and the curve flattens out.

EXPERIMENT II.—*To determine the rate of action of various dilutions of the bacteriophage.*

The three mixtures used had the following composition:

A bacteriophage 1/10, bacilli 3130 per drop, temperature 22° C.
B „ 1/100 „ 2464 „ „ 21·5° C.
C „ 1/200 „ 1900 „ „ 22·5° C.

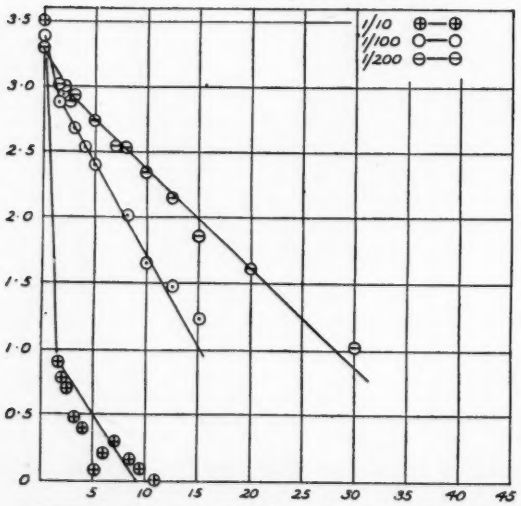

CHART 2.—The rate of action of various dilutions of bacteriophage. Ordinates = logs of number of surviving bacteria.

The results from these three experiments are combined in Chart II. The figures from which the chart is constructed are omitted for brevity.

In this case the logarithms of the numbers are plotted so as to get all the figures into one diagram.

Discussion of Chart II.—The bacteriophage diluted 1/10 reduced the bacilli from 3130 per drop to 8 per drop in 1½ minutes, *i. e.* 99·74 per cent. had been destroyed in that time.

The 1/100 dilution took 15

minutes to reduce the bacilli from 2464 to 16 per drop—a destruction of 99·3 per cent.

The 1/200 dilution took 30 minutes to reduce the bacilli from 1900 to 11 per drop—a destruction of 99·4 per cent.

The initial inoculation in each case was not the same, but the figures show that the 1/200 dilution takes approximately twice as long to reduce the number of bacilli in the same proportion as the 1/100 dilution, and the 1/100 dilution takes about ten times as long as the 1/10 dilution.

The greater the dilution, the less marked is the initial drop in the number of organisms.

In the second stage of the reaction following the initial great fall, the destruction of the bacilli proceeds regularly and the logarithms of the numbers fall on a straight line (5 to 15 minutes).

This period is succeeded by a final phase in which the rate of destruction falls off.

EXPERIMENT III.—*To investigate the effect of temperature on the rate of the reactions.*

In some early experiments the mixtures of bacilli and lysin were kept at 27° C. in a water-bath; when the reaction was found to proceed so rapidly an attempt was made to slow it down by working at a lower temperature.

At 20° C. both the initial fall and the second phase were somewhat less rapid.

An experiment was then carried out in the cold room and the results obtained given in Chart III, where the rate at 0° C. is compared with that found at 27° C. The bacteriophage was diluted 1/100 in each case and the inoculation of organisms approximately the same.

Discussion of Chart III.—The initial drop in the number of organisms is much less at 0° C.

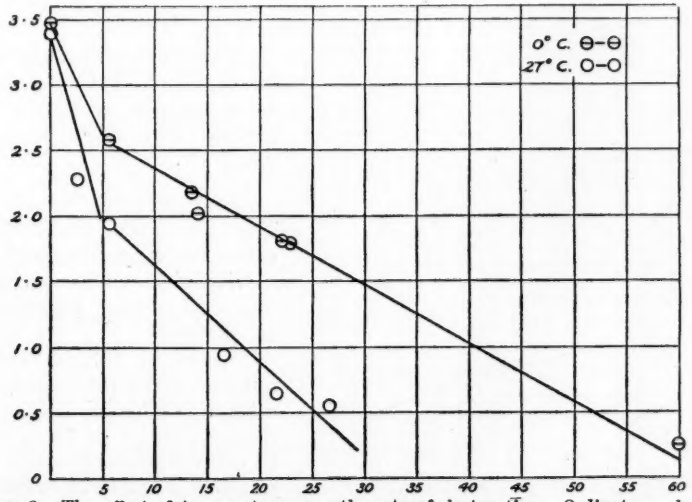

CHART 3.—The effect of temperature upon the rate of destruction. Ordinates = logs of number of surviving bacteria.

The rate is about half as rapid in the second stage.

Even at 0° C. the bacilli are reduced in number from 2935 to 2 per drop in one hour.

Some idea of the order of magnitude of the temperature-coefficient can be obtained by considering the figures of this experiment.

At 27° C. 2465 bacilli were reduced to 192, *i.e.* 7·8 per cent.—in 2·5 minutes. At 0° C., interpolating from the chart, 2935 bacilli were reduced to 229, *i.e.* 7·8 per cent. in 8·5 minutes. The rate being inversely as the time taken, it follows that—

$$\frac{\text{The rate at } 27° \text{ C.}}{\text{The rate at } 0° \text{ C.}} = \frac{8·5}{2·5} = \frac{3·4}{1}.$$

The temperature-coefficient for 10° C. calculated from these figures is 1·57

Considering Experiment II the 1/100 dilution of bacteriophage acting at 21·5° C. took 5·5 minutes to reduce 2462 bacilli to 192 (interpolated), so that—

$$\frac{\text{The rate at } 21·5° \text{ C.}}{\text{The rate at } 0° \text{ C.}} = \frac{8·5}{5·5} = \frac{1·55}{1}.$$

The temperature-coefficient for 10° C. from these data is 1·2.

EXPERIMENT IV.—*To compare the action of a 1/100 dilution of the bacteriophage on different numbers of organisms.*

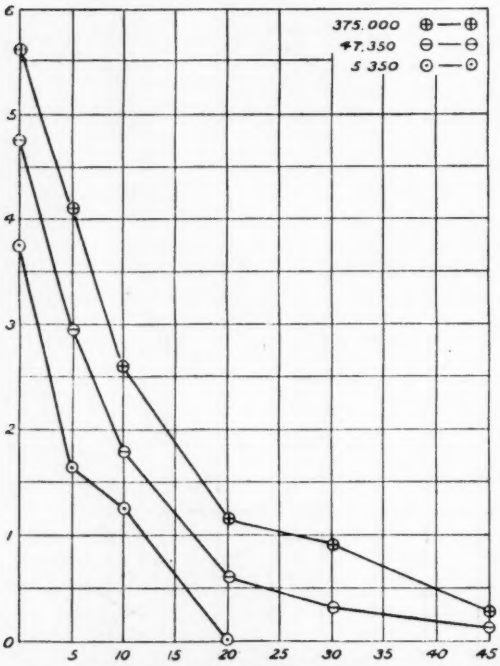

CHART 4.—The rate of action of bacteriophage in the presence of different numbers of organisms. Ordinates = logs of number of surviving bacteria.

Method used.—Three tubes, each containing 1/100 bacteriophage and the following number of organisms, were prepared:

1. 5,350 per drop.
2. 47,350 „
3. 375,000 „

These mixtures were kept on the bench at 15° C. and samples were removed at intervals and plated. The results are given in Chart IV.

Discussion of Chart IV.—The lines joining the logarithmic values of the numbers of bacilli surviving in each of the three mixtures at each period of time are parallel.

Hence it follows that the number of bacilli destroyed in a mixture of bacilli and any particular dilution of bacteriophage in a given time depends on the number of bacilli inoculated. At

the end of five minutes, for example, the number of bacilli killed in the three mixtures was as follows:

1. 5,313, or 99·3% of the inoculum.
2. 46,415, or 98·0% „ „
3. 370,928, or 98·8% „ „

General Discussion of the Rate and Course of the Reaction.

The points representing the numbers of surviving bacilli at different periods in the killing process appear to lie, when plotted, on a fairly smooth curve, but no satisfactory expression has been found for the complete reaction. It is clear from the plotted figures for the logarithms of the surviving bacteria that only the middle or a portion of the middle stage of the process proceeds in the manner of a monomolecular reaction.

The initial stage of lightning killing and the final stages cannot be so expressed, and it is just those stages (and particularly the first) which it is most desirable to be able to analyse. The final stage is complicated by technical difficulties in plating small numbers of residual organisms. When working with such dilutions as 1/100 bacteriophage the carrying over of more than a certain volume of the mixture to the amount of agar used in plating results in a concentration of lysin which can produce a lethal action on the residual bacilli.

With regard to the initial stages of the reaction, the logarithms of surviving bacteria in practically all the experiments cited in this paper and in others not cited lie completely outside any straight line representing a subsequent monomolecular phase. On further analysis, however, it is found that the initial stage finds fairly satisfactory expression by a formula of the bimolecular type in which the velocity of the reaction at any point is proportional to the square of the surviving bacteria; ($\frac{-dy}{dx} = ky^2$, where x = time and y = surviving bacteria).

The constant, k, is calculated from the expression $k = \frac{y_o - y}{xy}$, where y_o is the initial seeding.

In the following example (Table II) k has been so calculated and also k' obtained from the monomolecular formula $k' = \frac{1}{x - x_o} \log \frac{y_o}{y}$.

From the fact that a formula of the bimolecular type fits the initial figures fairly closely it would be futile, in the present state of knowledge, to draw any inference as to the nature of the reaction between phage and organisms based on physico-chemical principles. The apparent discontinuity of the reaction, however, suggests that the concentration of the effective substance is altering during the initial stage. With very strong bacteriophage (1/10 dilution), the whole reaction appears to proceed bimolecularly according to the simplified formula $xy = k$, the surviving bacilli at any point being negligible in comparison with the initial inoculum (*i.e.* $y_o - y$ is practically constant).

TABLE II.—*Bacteriophage diluted* 1/200, *Temperature* 22·5° C.

Time (x).	Bacilli (y).	k (bimolecular constant).		k' (monomolecular constant).	
0′ 0″	1900				
1′ 30″	1152	0·43			
2′	979	0·46		$x_0 = 2$, $y_0 = 979$	
2′ 30″	762	0·59		·216	
3′	860	0·40		·056	
4′	(402)	(0·90)	Mean k, 0·51	(·193)	
5′	548	0·49		·078	
7′	348	0·63		·09	
8′	339	0·57		·076	
10′	223	·75		·08	
12′ 30″	142	·991		·079	Mean k', 0·078
15′	73	1·76		·086	
20′	41·3	2·38		·077	
30′	10·7	6·3		·071	
62′	1·7	18·05		·046	

On the basis of the disinfectant experiments of Henderson Smith (1921) the discontinuity may be attributed simply to a very skew distribution of resistances in the population presented to the action of the lysin. The question of variation in resistance of the bacilli needs very special consideration in the mode of action of bacteriophage, as of necessity specially sensitive organisms are selected in order that a reaction may take place.

When a specially resistant type of the autogenous bacillus was selected from "nibbled" colonies and treated with the bacteriophage, there was a rapid killing off in the first three minutes of the most sensitive bacilli. After this initial effect there was no further reduction in the number of organisms.

The rapidity of action of bacteriophage recalls the similar phenomenon noted by Rettger and Sperry (1912) in connection with the bactericidal action of egg-white on certain susceptible organisms, and of Chick (1913) on the bactericidal properties of blood-serum, and of Fleming (1922) in connection with the bactericidal action of tears.

In the light of these results, further experiments were undertaken with the object of testing whether bacteriophage became absorbed in the course of the reaction.

EXPERIMENT V.—*To test the activity of a* 1/100 *dilution of bacteriophage immediately after it has acted on varying numbers of bacilli.*

Method A.

Four tubes each containing 4·9 c.c. of 1/100 bacteriophage were prepared. One was kept as a control; the others were inoculated with the following numbers of organisms:

(1) 2485. (2) 29,250. (3) 171,000.

The tubes were kept at 18° C. for 54 minutes. At the end of that time 1 c.c. was removed from each and from the control, and placed in tubes containing 4 c.c. of peptone-water, so that each sample was now diluted 1/500.

At the end of 64 minutes these 1/500 dilutions were plated, the average number of organisms surviving per drop being—

(1) 0·75. (2) 1·5. (3) 5.

At the end of 1 hour 25 minutes each tube received an inoculum of 1282 bacilli per drop. Plates were made at the end of 10, 22, 40 and 94 minutes. The results are shown in Table III.

TABLE III.

Time of plating.	Average number of bacilli per drop in different samples. Control.	(1).	(2).	(3).
0 min.	1282	1282	1282	1282
10 ,,	(1) 484 (3) 372 } mean 378	(1) 303 (2) 400 } 351	(1) 504 (1) 412 } 458	(1) 137 (1) 438 } 310
20 ,,	(2) 166 (5) 146 } 156	(1) 127 (2) 231 } 179	(1) 186 (2) 128 } 157	(1) 239 (2) 114 } 176
40 ,,	(2) 41 (10) 18·6 } 29·8	(2) 48·5 (5) 26·8 } 37·6	(2) 39 (5) 33 } 36	(1) 3 (3) 5·3 } 4

The number of drops plated are shown in brackets.

Summary of Table III.—Very little difference could be detected between the numbers of bacilli surviving in the different samples at the end of various periods; hence the slowing off of the reaction which takes place during the second phase cannot be explained on the supposition that lysin is adsorbed by the bacilli and put out of action.

Method B.

When the three different numbers of bacilli had been acted on by the bacteriophage for 50 minutes, the three samples, and a control 1/100 dilution of bacteriophage which had not been in contact with bacilli were sterilised by heating at 56° C. for one hour, and the experiment carried on as before. The results are shown in Table IV and Chart V.

TABLE IV.

Time of plating.	Samples of bacteriophage derived from Experiment VB: numbers of colonies per drop. Control.	1.	2.	3.	Control unheated.
0 min.	3170	3170	3170	3170	3170
17 ,,	631	1322	1686	2470	288
37 ,,	292	398	621	—	29·8
48 ,,	—	—	—	1475	—

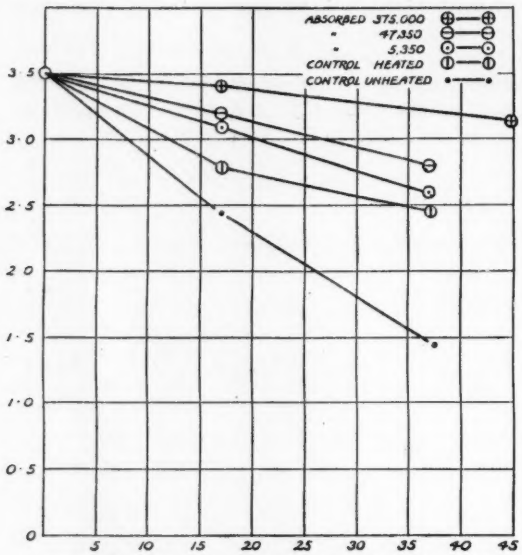

CHART 5.—The rate of action of bacteriophage which has acted on different numbers of organisms and been heated at 56° C. for 1 hour. Controls of heated and unheated bacteriophage which have not acted on organisms. Ordinates = logs of number of surviving bacilli.

Summary of Chart V.—Heating a dilution of 1/500 bacteriophage at 56°C. for one hour reduced its activity as compared with the unheated control.

The heated samples which had been in contact with bacilli showed an even greater loss of activity.

The reduction was greatest with the largest number of bacilli and least with the smallest number of organisms.

The activity of a given dilution of bacteriophage is not reduced by its action on varying numbers of bacilli; if, however, the lysin is heated in the presence of the dead bacilli a reduction in its activity can then be detected.

There is, therefore, no evidence that the slowing off of the rate which occurs during the second phase of the action is due to a disappearance of the lysin. It seems probable that it must be due to the higher resistance of the organisms left in the later stages of the reaction.

GENERAL CONCLUSIONS.

1. The rate of action of bacteriophage on sensitive bacilli is very rapid; 1 in a 100 dilution of the bacteriophage used in these experiments killed off 99 per cent. of the organisms inoculated in five minutes.

2. The rate of destruction of the remaining organisms is much slower; this is not due to a disappearance of the lysin, but is probably to be accounted for by a higher resistance on the part of the remaining bacilli.

3. The rate of the reaction depends on the concentration of the bacteriophage, a 1/200 dilution taking approximately twice as long to produce the same effect as a 1/100 dilution.

4. The number of bacilli destroyed by a given dilution of bacteriophage in a given time varies directly with the number of organisms inoculated.

5. The temperature co-efficient of the reaction is low, the rate at 0° C. being about one-third that at 27° C.

6. Heating bacteriophage at 56° C. for one hour reduces its activity; if bacilli which had been acted on by the lysin during the heating this reduction in activity is more marked,

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STUDIES IN EXPERIMENTAL TETANY: I. DISTRIBUTION OF CALCIUM. II. COLLOIDAL AND IONIC CALCIUM.

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THE DISTRIBUTION OF CALCIUM IN THE PLASMA AND CELLS.

NUMEROUS estimations upon the total calcium of blood, of plasma and of serum have been carried out, and while a certain divergence of results still exists, yet the general average arrived at by various workers is practically the same. In their earlier work MacCallum and Voegtlin stated that the percentage of calcium in whole normal blood was about 13·3 mg. while in tetany blood it was about 5·4 mg. Several years later these workers by means of a different analytical method arrived at the conclusion that their former results were too high, and that more correct figures for the percentage of calcium in normal and tetany blood would be 6·1 mg. and 2·7 mg. respectively.

Variations in results are undoubtedly due to difference in the methods used for the estimation of blood calcium. Of the two methods more generally used in recent years, namely, those of Lyman (1917) and Halverson and Bergheim (1917), it seems that the experimental error is much more under control with the former than with the latter. A nephelometric method which depends upon the accurate matching of shades or depth of colour is always open to a personal error, and such method cannot safely be adopted until by practice

he who uses it is assured by stringent tests that his readings are within 0·5 to 1 mm. of the correct figure.

In the present investigation Lyman's method has been used and has proved very satisfactory for estimating small amounts of calcium. The nephelometer used was made by taking a Duboscq colorimeter, replacing the cups and plungers with hard rubber cylinders into which short prepared test-tubes fitted accurately.

The test-tubes were supported by means of their flanged edges resting in two lateral slits cut into a hard rubber plate which was screwed on to the colorimeter beneath the prism. The cylinders, supported by movable rings resting on the cup supports, were so adjusted that when the tops were in contact with the hard rubber plate, thereby excluding all light, the indicators on the millimetre scale stood at the zero mark.

The nephelometer attachment was made of wood painted black on the outside and white within. The partition placed between the lamps and the colorimeter contained a plate of frosted glass, and the illumination consisted of one set of two 100-c.p. 110-volt lamps and one set of two 100-c.p. 220-volt lamps, so arranged that any number could be used for illumination.

In determining the distribution of calcium in plasma and cells, it was necessary to obtain accurate values for the proportion of cells to plasma in whole blood. For this purpose it was found that the ordinary hæmatocrit was not satisfactory and the following method was adopted. A piece of pressure glass tubing 10 cm. long having an internal diameter of 1 mm. and a wall thickness of 3 mm. was taken and the ends made smooth with a file. To a 50 c.c. metal centrifuge holder a small right-angled piece of iron was attached so that it formed an inverted L, the arm of the letter being placed over the mouth of the cup and having a tapped hole at the distal end, placed accurately over the centre of the cup. The glass tube filled with blood was placed in a stout rubber band; a small brass cup was placed over the upper end of the tube, the lower end of which was then pressed firmly into the rubber cushion in the bottom of the centrifuge cup by means of a nut which, passing through the tapped hole, fitted into a narrow and comparatively deep hole in the brass cup.

This device was used in duplicate, and after centrifuging the specimen of blood for fifteen minutes very accurate measurements could be made of the proportion of cells to plasma in a length of 10 cm. of blood.

From Table I it will be seen that the average result of the total calcium of the blood of fourteen dogs is 9·12 mg. per cent. In computing this average the figures for Dogs Nos. VIII and IX are not included, as these dogs were suffering severely from mange.

The average figure for the amount of calcium in plasma from 100 c.c. of blood is 8·11 mg., while for the corpuscles from the same amount of blood it is 1·01 mg.—that is to say, in a hundred parts by volume of blood we find a distribution of calcium between the plasma and the cells in a proportion aproximately of 8 to 1.

In a series of some twelve rabbits in which similar estimations were made it was found that the figures were slightly higher, being for whole blood 10·0 mg. per cent., for plasma from 100 c.c. of blood 8·6 mg., and for the cells 1·4 mg.

With the parathyroidectomised dogs estimations were made usually on the second day after operation. If the animals survived for two or three days longer, then a second determination was made. On several of these animals a partial parathyroidectomy was performed, but no conclusive results were obtained immediately following such an operation, and the results quoted are those in totally thyro-parathyroidectomised animals. The pH was estimated on the day following operation on blood drawn from the left ventricle, the method used being that of Levy, Rowntree and Marriott (1915).

TABLE I

DISTRIBUTION OF CALCIUM IN THE CELLS AND PLASMA OF NORMAL DOGS.

DOG	pH	TOTAL Ca mgs. %	HÆMATOCRIT READING mm.	PLASMA Ca PER 100 cc. BLOOD	CELL Ca PER 100 cc BLOOD	CELLULAR Ca % OF TOTAL
I	7·30	9·60	3·7	8·36	1·24	12·19
II	7·30	8·10	3·6	7·20	·90	11·1
III	7·30	8·00	8·4	7·36	·74	9·3
IV	7·30	8·00	2·8	7·28	·72	9·0
V	7·35	7·75	3·4	6·79	·96	12·2
VI	7·35	8·10	3·1	7·24	·86	11·6
VII	7·35	10·00	3·1	8·83	1·17	11·7
VIII	7·30	8·20	3·2	7·80	·40	4·9
IX	7·20	7·50	3·2	7·20	·30	4·0
X	7·30	7·85	3·5	6·95	·90	10·2
XI	7·25	9·12	3·5	8·25	1·87	9·6
XII	7·25	9·60	3·0	8·40	1·20	12·5
XIII	7·25	10·80	2·7	9·56	1·24	11·5
XIV	7·30	11·00	3·4	9·90	1·10	10·0
XV	7·30	9·70	2·8	8·85	·85	8·8
XVI	7·30	10·00	2·5	8·70	1·30	13·0

TABLE II

DISTRIBUTION OF CALCIUM IN THE CELLS AND PLASMA OF TETANY DOGS.

DOG	pH	TOTAL Ca mgs. %	HÆMATOCRIT READING mm.	PLASMA Ca PER 100 cc. BLOOD	CELL Ca PER 100 cc. BLOOD	CELLULAR Ca % OF TOTAL
I	7·40	5·82	3·0	5·32	·50	8·5
II	7·50	5·37	1·9	4·86	·51	9·5
III	7·45	5·62	2·8	5·19	·43	7·6
IV	7·53	5·75	3·2	5·16	·51	10·26
V	7·40	6·50	8·5	6·04	·46	7·1
VI	7·50	6·25	3·3	5·83	·42	6·7
VII	7·40	6·50	3·6	6·10	·42	6·2
X	7·45	4·93	3·5	4·45	·48	9·7
XI	7·45	4·75	2·7	4·41	·34	7·1

NORMAL DOGS AVERAGE pH = 7·293
TETANY DOGS AVERAGE pH = 7·455
pH DIFFERENCE 0·162

TABLE III

COMPARISON OF AVERAGE FIGURES FOR CALCIUM DISTRIBUTION IN NORMAL DOGS AND TETANY DOGS.

Ca % IN mgs. WHOLE BLOOD	PLASMA Ca mgs. PER 100 cc OF BLOOD	PLASMA Ca % OF TOTAL CALCIUM	CELL Ca PER 100 cc BLOOD	CELL Ca % OF TOTAL Ca	REMARKS
9·12	8·11	88·9	1·01	11·1	NORMAL DOGS
5·72	5·26	91·9	·46	8·1	TETANY DOGS
37·4	35·2		54·4		PERCENTAGE FALL IN Ca

Taking seven completely thyro-parathyroidectomised dogs, the average figures, quoted in Table II, show a whole blood calcium content amounting to 5·72 mg. per cent., with a plasma and cell content per 100 c.c. of whole blood of 5·26 mg. and 0·46 mg. respectively. It is thus seen that in experimental tetany the ratio of plasma calcium to cell calcium is approximately 11·5 to 1·0.

The alteration in the calcium distribution may be more clearly seen from Table III, in which the relative percentage of calcium in the plasma and cells to the total blood calcium is given, and in which the comparative alterations are indicated as percentages of the normal contents. It is here shown that in the condition of tetany there is a considerable loss of cell calcium amounting to 54·4 per cent. as compared with a plasma loss of 35·2 per cent.

DIFFUSIBLE AND COLLOIDAL CALCIUM IN NORMAL AND THYRO-PARATHYROIDECTOMISED DOGS.

In 1916 a method for the estimation of diffusible calcium was introduced by von Mysenbug and others (1916). This method is one of compensation dialysis, in which the diffusible salt in the dialyser is balanced by the addition of an equal amount of the salt to the dialysing fluid.

The separation by filtration of the diffusible salts of blood-serum can be effected by subjecting the filter to a pressure of 150 mm. of Hg. This method was employed by Cushny (1920); but to determine accurately the amounts of a salt in ionic and colloidal form it is essential not to alter the balance between them by removal from the solvent of the diffusible salt. According to the law of mass action the removal of the ionic calcium would result in a further breakdown of calcium in combination in order to restore the normal balance. For this one reason, therefore, apart from others, the compensation method of dialysis is the better and has been made use of in this work.

While a considerable drop in the total calcium of the blood and of its cellular elements has been noted in parathyroid tetany, the question as to whether or not this was associated with a loss in free or combined calcium has not been definitely settled. That alterations may occur in the diffusible calcium in rickets and in tetany was suggested by von Mysenbug and his co-workers, but in a series of four experiments they found no distinct variation from the normal.

METHODS.

Preparation of parlodion sacs.—The shreds of parlodion are dried in the air and dissolved in ether and 95 per cent. alcohol in the required proportions. After solution of the parlodion shreds is complete, the bottle, firmly stoppered, is allowed to stand for two weeks.

Two small test-tubes, 15 mm. internal diameter and 60 mm. long, are filled with the mixture, centrifuged to bring all air-bubbles to the surface, the hardened surface then peeled off and the contents emptied into the stock bottle, the tube being rotated meanwhile in order to secure uniform thickness of the wall of the sac. The tube is clamped in the inverted position for 15 minutes, then placed in cold distilled water for half-an-hour. The upper rim is loosened by means of a knife, and a thin glass rod with a bulbous tip gently pushed between the sac-wall and the glass to the bottom of the test-tube, which is kept half filled with water. The sac and the glass rod are withdrawn together and the sac immersed in cold distilled water.

Compensation dialysis.—In view of the fact that in paratyphoid tetany variations in diffusible calcium may be great or small, it was necessary to make up a series of dialysing calcium solutions, in order to balance as nearly

as possible the amount of free calcium found in the serum. For this purpose ten dialysing solutions were made up, giving a percentage of normal serum calcium varying from 40 to 80. These solutions were made from a stock solution of calcium chloride of about 1 per cent. strength, the amount of calcium in the stock solution having been determined by the nephelometric method. The following is the series of solutions employed:

COMPENSATION DIALYZING SOLUTIONS.

SOLUTION	AMOUNTS OF Ca. IN mgs. IN 2·5 cc.		AMOUNTS OF Ca. IN mgs. %		PER CENT. OF NORMAL Ca.
	ESTIMATED	CALCULATED	ESTIMATED	CALCULATED	
A	·206	·193	8·25	7·71	39·28
B	·213	·210	8·54	8·40	40·65
C	·243	·241	9·75	9·65	46·42
D	·257	·262	10·30	10·30	49·10
E	·290	·289	11·62	11·58	55·33
F	·312	·315	12·50	12·60	59·52
G	·343	·338	13·75	13·50	65·47
H	·367	·363	14·68	14·70	69·90
I	·390	·355	15·62	14·19	74·38
J	·406	·420	16·25	16·70	77·38

To 2·5 c.c. of the solution employed 2·5 c.c. of a double strength dialysing fluid were added, the composition of which is as follows:

DIALYZING FLUID: (GREENWALD)

SALT	PER CENT AMOUNTS SINGLE STRENGTH	MOLAR STRENGTH	AMOUNTS IN 2000cc. DOUBLE STRENGTH
$NaHCO_3$	·2520 gm.	·003	10·08 gm.
KH_2PO_4	·0088 gm.	·000065	12·3 cc M/5
$MgCl_2$	·01059 gm.	·00011	0·2118 gm.
KCl	·02960 gm.	·000397	1·184 gm.
NaCl	·6250 gm.	·01069	25·0 gm.

Since the balance between diffusible and colloidal calcium is in some measure dependent upon the H-ion concentration of the serum, it is essential to bring the CO_2 tension of the serum into equilibrium with an atmosphere of CO_2 of a tension approximating that of alveolar air. To do this a large spirometer was filled with CO_2 and air; these were thoroughly mixed, and the percentage of CO_2 estimated daily by the Henderson and Morris (1917) method.

The gas was passed through a bottle containing moist glass beads, then through two separatory funnels placed in series, the first containing about 20 c.c. of serum, the second about 10 c.c. of the double strength dialysing fluid. This equilibration was carried out at room temperature, which during the course of these experiments varied from 30° to 33° C.

The dialysis.—The sacs to be employed were thoroughly washed in distilled water, shaken free of water and allowed to drain, but not allowed to dry in the air. The test-tubes used for dialysis were $\frac{3}{4}$ in. wide by 4 in. long, and into these were placed 2·5 c.c. of the double strength dialysing fluid, and CO_2 passed into the test-tubes for a few minutes.

The sacs, supported on small hard rubber cylinders, were filled with 5 c.c. of serum, CO_2 was passed into the sacs, a small cork firmly inserted into the top of each cylinder, and the whole supported in the test-tube. Dialysis was carried out for 24 hours at a temperature of 12° to 15° C.

DISCUSSION OF RESULTS.

In the following series of tables, Nos. IV to VII, the results obtained in normal, partially, and totally thyro-parathyroidectomised dogs are given.

TABLE IV.

TOTAL SERUM CALCIUM IN NORMAL DOGS, IN PARTIALLY, AND IN COMPLETELY PARATHYROIDECTOMIZED DOGS.

NORMAL DOGS	DOGS PARTIALLY PARATHYROIDECTOMIZED.	DOGS COMPLETELY THYRO-PARATHYROIDECTOMIZED.
11·24	6·25	5·00
10·50	6·13	5·10
11·20	8·20	5·00
11·30	AVERAGE 7·04 mgs.	4·70
12·70		5·20
12·60		4·60
9·00		5·40
AVERAGE 11·30 mgs. (100%)	(DROP = 37·72%)	AVERAGE 4·95 mgs. (DROP = 56·20%)

Table IV shows the variations in percentage of the calcium content of serum under the three specified conditions. From a normal average of 11·30 mg. per cent., the calcium fell in partially parathyroidectomised animals to an average of 7·04 mg. per cent., to be followed by a further fall upon complete parathyroidectomy to an average figure of 4·95 mg. per cent. It is thus seen that the loss of calcium from the serum amounts to 56·20 per cent. of the whole.

With regard to diffusible calcium the figures quoted in Table V, comprising results obtained from a series of seven normal dogs, are similar to those of Cushny and von Mysenbug, namely that in normal animals the diffusible calcium amounts to between 60 and 70 per cent. of the whole calcium content of the blood, taking 10·5 mg. as the percentage of calcium in normal blood-serum.

In comparing the results recorded in Tables VI and VII from partially and completely thyro-parathyroidectomised dogs respectively, it is seen that there is no essential difference in the amounts of calcium recorded.

It is somewhat difficult to say to what extent a single parathyroid gland may or may not be functioning. The upper left parathyroid was left with a small piece of thyroid tissue. In all of these cases the gland retained was distinctly identified, but we must admit that this method of leaving a gland *in sitû*, while all other similar tissue is removed, is not, *par excellence*, the method that should be followed in the investigation of the internal secretion of any gland. Just as in partial depancreatisation, so in partial parathyroidectomy, the gland or piece of gland should be transplanted and the activity of the graft definitely proved before it is totally removed. In the former method one cannot, with assurance, state whether or not there has been any alteration in the normal blood- and lymph-supply due to operative interference. In all of these operative animals subcutaneous suture was employed, no dressings were applied, and without exception excellent healing of the neck wounds was obtained.

From the average percentage of diffusible calcium recorded for these tetany animals, namely, 93·70, the individual percentage being computed from the total amount of calcium in each sample of serum, it is evident that the loss of colloidal calcium is indeed a marked one.

It is true that the compensation in dialysing was not complete; but in view of the high percentage recorded, and also in view of the result obtained with the final serum from Dog VIII, in which no compensation was attempted, one is forced to the conclusion that in severe tetany the major part, if not all, of the colloidal calcium is freed from its protein combination. It would also seem from certain of the results that it would be possible to determine the rate of the breakdown of this calcium proteinate linkage.

RESULTS OF COMPENSATION DIALYSIS OF SERUM CALCIUM.

TABLE V. NORMAL DOGS.

DOG I. TOTAL Ca = 10·5 mgs. %: Ca IN 5 cc. SERUM = 0·525 mgs.: CO_2 TENSION = 49·64 mm. Hg.

Ca IN mgs. IN 5 cc. SERUM		Ca IN mgs. IN 5cc. DIALYZATE		DIFFUSIBLE CALCIUM PER CENT.	COMPENSATION DIALYZING SOLUTION
BEGINNING	END	BEGINNING	END		
·525	·584	·343	·317	55·6	G
·525	·534	·343	·316	55·4	G
·525	·493	·312	·332	67·1	F
TOTAL Ca = 11·24 mgs. %:		Ca IN 5cc. SERUM = 0·562 mgs.:		CO_2 TENSION = 50·16 mm. Hg.	
·562	·503	·312	·337	64·4	F
·562	516	·343	·413	61·4	G
·562	·462	·367	·383	70·6	H

DOG II. TOTAL Ca = 10·5 mgs. %: Ca IN 5cc. SERUM = 0·525 mgs.: CO_2 TENSION = 52·92 mm. Hg.

·525	·530	·343	·344	65·5	G
·525	·515	·367	·354	64·9	H

DOG III. TOTAL Ca = 11·7 mgs. %: Ca IN 5cc. SERUM = 0·585 mgs.: CO_2 TENSION = 50·92 mm. Hg.

·585	·505	·312	·342	63·5	F
·585	·558	·343	·362	65·1	G
·585	·550	·367	·382	67·9	H
·585	·557	·367	·377	66·1	H

DOG IV. TOTAL Ca = 11·30 mgs. %: Ca IN 5cc. SERUM = 0·565 mgs.: CO_2 TENSION = 44·14 mm. Hg.

·565	·480	·312	·343	66·1	F
·565	·491	·343	·323	59·1	G
·565	·511	·367	·362	63·1	H
·565	·537	·367	·372	66·7	H

DOG V. TOTAL Ca = 12·7 mgs. %: Ca IN 5cc. SERUM = 0·635 mgs.: CO_2 TENSION = 42·56 mm. Hg.

·635	·580	·312	·362	64·8	F
·635	·571	·343	·363	60·1	G
·635	·550	·367	·380	62·0	H
·635	·550	·367	·387	64·0	H

DOG VI. TOTAL Ca = 12·6 mgs. %: Ca IN 5cc. SERUM = 0·630 mgs.: CO_2 TENSION = 50·0 mm. Hg.

·630	·590	·312	·332	55·9	F
·630	·589	·343	·352	36·8	G
·630	·621	·367	·372	59·3	H
·630	·599	·367	·367	57·8	H

DOG VII. TOTAL Ca = 9·0 mgs. % Ca IN 5cc. SERUM = 0·450 mgs.: CO_2 TENSION = 49·4 mm. Hg.

·450	·470	·312	·312	69·3	F
·450	·481	·367	·342	70·4	H

PARTIALLY THYRO-PARATHYROIDECTOMIZED DOGS.

TABLE VI.

DOG I. TOTAL Ca = 6·75 mgs. %: Ca IN 5cc. SERUM = 0·337 mgs.: CO_2 TENSION = 50·0 mm. Hg.

Ca IN mgs. IN 5 cc. SERUM		Ca IN mgs. IN 5cc. DIALYZATE		DIFFUSIBLE CALCIUM PER CENT.	COMPENSATION DIALYZING SOLUTION
BEGINNING	END	BEGINNING	END		
·337	·340	·213	·250	80·0	B
·337	·368	·257	·291	96·4	D
·337	·363	·312	·296	83·1	F
·337	·368	·367	·352	100·0	H

DOG II TOTAL Ca = 6·13 mgs. %: Ca IN 5cc. SERUM = 0·306 mgs.: CO_2 TENSION = 47·83 mm. Hg.

·306	·335	·257	·302	103·5	D
·306	·302	·257	·272	93·2	D
·306	·352	·312	·393	90·0	F
·306	·291	·406	·372	109·0	J

DOG III TOTAL Ca = 8·2 mgs. %: Ca IN 5cc. SERUM = 0·410 mgs.: CO_2 TENSION = 48·64 mm. Hg.

·410	·410	·257	·281	84·5	D
·410	·391	·367	·392	101·6	H
·410	·391	·406	·412	101·9	J

COMPLETELY THYRO-PARATHYROIDECTOMIZED DOGS.

TABLE VII

DOG I. TOTAL Ca = 5·0 mgs.%: Ca IN 5cc. SERUM = 0·250 mgs.: CO_2 TENSION = 49·4 mm. Hg.

JULY 21. OPERATION. JULY 22. HYPERPNŒA OF CHEYNE-STOKES TYPE, WITH SALIVATION, FINE TREMOR OF MUSCLES, TETANY NOT SEVERE.

JULY 22. 70 cc. OF BLOOD DRAWN FROM FEMORAL ARTERY.

Ca IN mgs. IN 5cc. SERUM		Ca IN mgs IN 5cc. DIALYZATE		DIFFUSIBLE CALCIUM PER CENT.	COMPENSATION DIALYZING SOLUTION
BEGINNING	END	BEGINNING	END		
·250	·314	·312	·280	99·2	F
·250	·246	·257	·221	90·0	D
·250	·310	·367	·312	102·8	H
·250	·291	·406	·332	95·2	J

DOG II. JULY 20. COMPLETE OPERATION AT NOON. JULY 21. 2 P.M., TREMORS OF NECK AND SHOULDER MUSCLES, INCREASED RESPIRATION AWITH SLIGHT SALIVATION. 8 P.M., MARKED TREMORS OF MUSCLES OF THE NECK AND OF THE HIND LIMBS. RESPIRATIONS VERY RAPID AND OF CHEYNE-STOKES TYPE. 9 P.M., 75 cc. OF BLOOD DRAWN ASEPTICALLY FROM THE FEMORAL ARTERY UNDER ANÆSTHESIA. 10 P.M., ANIMAL MUCH IMPROVED; ABLE TO STAND AND ABLE TO DRINK ON ACCOUNT OF REDUCED RESPIRATORY RATE: ANIMAL MENTALLY ALERT.

TOTAL Ca = 6·08 mgs.%: Ca IN 5cc. SERUM = 0·305 mgs.: CO_2 TENSION = 41·8 mm. Hg.

·305	·279	·257	·272	69·0	D
·305	·273	·312	·291	90·0	F
·305	·273	·367	·322	90·3	H

JULY 22. DOG IN MARKED TREMOR, CAPABLE OF STANDING BUT LEGS STIFF: RESPIRATION VERY IRREGULAR AND RAPID WITH A RATE OF OVER 200 PER MINUTE: 70 cc. OF BLOOD TAKEN ASEPTICALLY UNDER ETHER: RESPIRATION RATE NORMAL UNDER ETHER: DEFINITE IMPROVEMENT FOR 36 HOURS AFTER WITHDRAWAL OF BLOOD, THE DOG BECOMING QUITE LIVELY AND RESPONSIVE. TREMORS COMMENCED WITHIN 36 HOURS, BEING FELT OVER THE SHOULDER GIRDLE: RESPIRATION RATE AGAIN RAPIDLY RISING FROM 60 PER MINUTE TO 180 PER MINUTE.

TOTAL Ca = 5·1 mgs.%: Ca IN 5cc. SERUM = 0·255 mgs.: CO_2 TENSION = 44·84 mm. Hg.

·255	·287	·312	·261	82·0	F
·255	·299	·406	·322	93·4	J

DOG III. TOTAL THYRO-PARATHYROIDECTOMY PERFORMED JULY 21: DOG DIED IN THE NIGHT ABOUT 20 HRS. AFTER OPERATION, HAVING DEVELOPED HYPERPNŒA AND SLIGHT TREMOR DURING THE EVENING OF THE 21ST: UNABLE TO OBTAIN FROM VEINS BECAUSE OF CLOTTING.

DOG IV. JULY 20: TOTAL THYRO-PARATHYROIDECTOMY.

JULY 26: THIS DOG HAS SHOWN NO SIGNS OF TETANY.

TOTAL Ca = 8·3 mgs.%: Ca IN 5cc. SERUM = 0·415 mgs.: CO_2 TENSION = 51·68 mm. Hg.

·415	·358	·367	·332	72·4	H
·415	·425	·257	·262	64·4	D

AUGUST 5: NO SIGNS OF TETANY HAVE DEVELOPED. TOTAL CALCIUM = 7·5 mgs.%.

DOG V. JULY 22: TOTAL THYRO-PARATHYROIDECTOMY PERFORMED: DOG IN EXCELLENT CONDITION.

JULY 23: SLIGHT HYPERPNŒA, MUSCULAR TWITCHING, AND SALIVATION DEVELOPING.

JULY 24: ALL THE MUSCLES OF THE ABDOMEN, SHOULDER, HEAD AND NECK AND LEGS IN CONSTANT AND RAPID TWITCHING. DOG IS VERY THIRSTY BUT UNABLE TO DRINK BECAUSE OF THE VERY RAPID RESPIRATION RATE AMOUNTING 240 PER MINUTE: THE DOG IS ABLE TO STAND, IS CONSTANTLY ON THE MOVE AND CONSTANTLY ATTEMPTING TO DRINK.

JULY 24: 10 A.M. BECAUSE OF THE SEVERITY OF THE CONDITION 70 cc. OF BLOOD WERE DRAWN FROM THE FEMORAL ARTERY. AT 2 P.M. THE CONDITION HAD GREATLY IMPROVED. ALL TREMORS HAD GONE AND RESPIRATION WERE ALMOST NORMAL.

JULY 26: NOON: TETANY ATTACK DEVELOPING WITH RAPID RESPIRATIONS. SALIVATION, MUSCLE TREMORS AND SPASTICITY OF LIMBS.

JULY 27: 9 A.M. SEVERE TETANY HAS DEVELOPED WITH STRONG SPASMODIC CONTRACTIONS OF MUSCLES OF THE HEAD AND JAW, WITH SHARP SNAPPING OF THE TEETH TOGETHER, CAUSING THE TONGUE TO BE BITTEN. RESPIRATION RATE, 250 PER MINUTE: SALIVATION AND THIRST MARKED: ANIMAL LYING SOAKED IN PERSPIRATION.
11 A.M. BECAUSE OF THE DISTRESSING CONDITION ANIMAL BLED TO DEATH.

COMPLETELY THYRO-PARATHYROIDECTOMIZED DOGS.

TABLE VII (CONT).

DOG V. (CONT.)

JULY 24: TOTAL Ca = 5·0 mgs. %: Ca IN 5cc. SERUM = 0·250 mgs.: CO_2 TENSION = 47·12 mm Hg.

Ca IN mgs. IN 5 cc. SERUM		Ca IN mgs. IN 5cc. DIALYZATE		DIFFUSIBLE CALCIUM PER CENT.	COMPENSATION DIALYZING SOLUTION
BEGINNING	END	BEGINNING	END		
·250	·313	·257	·254	100·4	D
·250	·313	·312	·282	100·8	F

JULY 26: TOTAL Ca = 4·7 mgs. %: Ca IN 5cc. SERUM = 0·235 mgs.: CO_2 TENSION = 46·36 mm. Hg.

·235	·256	·257	·246	100·0	D
·235	·268	·312	·271	97·8	C

JULY 27: TOTAL Ca = 4·7 mgs. %: Ca IN 5cc. SERUM = 0·235 mgs.: CO_2 TENSION = 42·56 mm. Hg.

·235	·305	·312	·227	96·6	F
·235	·332	·367	·300	99·1	H

DOG VI. JULY 22: TOTAL THYRO-PARATHYROIDECTOMY PERFORMED.

JULY 30: USUAL SIGNS OF TETANY; 70 cc. OF BLOOD TAKEN FROM FEMORAL ARTERY FOR ESTIMATIONS AND ANIMAL BLED TO DEATH.

TOTAL Ca = 4·6 mgs. %: Ca IN 5cc. SERUM = 0·230 mgs.: CO_2 TENSION = 50·16 mm. Hg.

·230	—	·312	·263	93·1	F
·230	·322	·367	·284	87·4	H

DOG VII. JULY 29: TOTAL THYRO-PARATHYROIDECTOMY PERFORMED: VERY DEFINITE SIGNS OF TETANY DEVELOPED WITHIN 48 HOURS.

TOTAL Ca = 5·2 mgs. %: Ca IN 5cc SERUM = 0·260 mgs.: CO_2 TENSION = 50·16 mm. Hg.

·260	·269	·257	·251	94·3	D
·260	·307	·367	·312	98·8	H

DOG VIII. AUG. 2: NOON. TOTAL THYRO-PARATHYROIDECTOMY PERFORMED.

AUG. 3: 9 A.M. MARKED TREMOR IN MUSCLES OF THE HEAD AND NECK, SHOULDERS, ABDOMEN: RESPIRATION RATE, 220 PER MINUTE: 70 cc. OF BLOOD TAKEN FROM FEMORAL ARTERY FOR ESTIMATIONS.

AUG. 4: CONDITION RELIEVED TEMPORARILY BY WITHDRAWAL OF 70 cc. OF BLOOD.

AUG. 5: 2 P.M. TREMORS OF MUSCLES VERY MARKED: PERSPIRATION AND SALIVATION MARKED: AT 7 P.M. TREMORS OF MUSCLES AS AT 2 P.M.: HÆMORRHAGE FROM THE TONGUE DUE TO INJURY FROM SPASMODIC CONTRACTION OF JAW MUSCLES: PERSPIRATION PROFUSE: SPASTICITY OF LIMBS MARKED: ANIMAL UNABLE TO STAND: CONDITION VERY SEVERE.

AUG. 3: TOTAL Ca = 4·6 mgs. %: Ca IN 5cc. SERUM = 0·230 mgs.: CO_2 TENSION = 50·16 mm. Hg.

·230	·286	·312	·241	73·5	F
·230	·290	·312	·240	73·0	F

AUG. 4: TOTAL Ca = 6·0 mgs. %: Ca IN 5cc. SERUM = 0·304 mgs.: CO_2 TENSION = 51·88 mm. Hg.

·230	·310	·312	·302	97·6	F
·230	·309	·367	·383	98·5	H

AUG. 5: TOTAL Ca = 5·4 mgs. %: Ca IN 5cc. SERUM = 0·270 mgs.: CO_2 TENSION = 49·56 mm Hg.

·270	·179	0	·120	98·8	NO COMPENSATION FLUID USED
·270	·138	0	·126	93·3	

THE PERCENTAGE OF CALCIUM WHICH IS DIFFUSIBLE CALCIUM IS CALCULATED AS FOLLOWS:

$$\text{DIFFUSIBLE CALCIUM} = \frac{\text{Ca IN DIALYZATE AFTER DIALYSIS} \times 2 \text{ MINUS Ca ADDED.}}{\text{ORIGINAL CALCIUM IN SERUM.}}$$

It appears, however, that the rapid loss of diffusible calcium from the blood demands a correspondingly rapid liberation of calcium from its colloidal state.

The great demand of the organism for calcium one can understand upon witnessing the severity of the condition. It seems feasible, therefore, to postulate that there is an attempt on the part of the organism to supply the bivalent salts necessary for the maintenance of a normal condition of irritability of the nervous system, and the method adopted is that of a rapid liberation in ionic form of all the colloidal calcium in the blood. Unfortunately, in virtue of its free state in the circulation, the calcium is very readily

lost by way of the kidneys, which are unable to respond properly to the abnormal condition by setting up a mechanism of curative adaptation. Under these conditions it is evident that once the store of colloidal calcium in the blood is utilised there must be a call upon the calcium of the tissues. That there is a muscle-protein breakdown in tetany has been amply proved by several workers, most notably by Paton, Findlay and Burns (1914–15), and Paton and Findlay (1916). It is possible, therefore, that there is a continual breakdown of muscle-protein, which is responsible for the production of a toxic substance, and it is this steady decomposition of protein which is the mechanism whereby an attempt is made to meet the call of the nervous and other tissues for calcium. In view of the urgent needs of the organism, and probably as a result of the protein decomposition, the calcium is thrown out in that form in which it is most easily utilised.

In considering the reason for the presence of diffusible calcium in the blood in tetany, the somewhat contradictory facts that both hydrochloric acid and calcium salts are effectual in the treatment of tetany open up a suggestion that the increase in the H-ion concentration of the blood during acute tetany may be a means whereby more calcium can be liberated from protein combination. The lessened alkalinity of the blood would favour the dissolving of the calcium, and would thereby make it immediately available for the body as soon as it was broken from its protein combination.

It must not be forgotten that the alteration in calcium metabolism is a sign of tetany, and that the causative factor is probably a body associated with the creatine of muscle protein.

The signs of parathyroid tetany.—In all these dogs, with the single exception of No. IV, definite symptoms of tetany developed. It is evident from the figures for Dog IV that there was no derangement of the free and combined calcium balance, but it will be noted that there is a slight though definite loss in total calcium.

Dogs II, V, VI, VII and VIII developed signs of tetany within thirty-six hours, and it will be seen from the notes in the tables that the attacks were, with only two exceptions, of a severe type. None of these animals suffering from severe tetany survived for more than six days. The signs noted as definitely indicating parathyroid tetany were the increased respiratory rate of from 200 to 250 per minute, with noisy expiration, often of a Cheyne-Stokes type, the profuse salivation and nasal secretion, all of which developed within forty-eight hours. The characteristic muscle tremors were invariably in evidence within twenty-four hours as fine muscle twitchings which could be felt most clearly over the shoulder girdle. These tremors became more and more marked until, in some cases by the end of the second day after operation, they had become coarse spasmodic twitchings. These were markedly in evidence in Dogs V, VII and VIII, in which cases the spasmodic contractions of the temporal and masseter muscles were undoubtedly distressing, the latter causing injury to the tongue by the sudden snapping together of the teeth.

The spasticity of the limbs, on account of which the animal is forced to lie on its side, the inability to stand, the cardiac irregularities, vomiting, loss of appetite, and at times profuse perspiration, were all in evidence two days, at the latest three days after operation.

The mild or more chronic types of tetany described by other observers in which the animals lived for two or three weeks after operation have not been seen throughout this investigation. All of ours were definite cases of tetany, most of them severe, terminating fatally within one week. The severity of these attacks was always greatly ameliorated by the withdrawal of the 70 c.c. of blood for the necessary examinations.

The severe condition occasioned by complete removal of the thyro-parathyroid apparatus may well account for the results recorded.

SUMMARY.

(1) The calcium content of 100 c.c. of normal blood amounts to the following average figures: Total 9·12 mg., plasma 8·11 mg., cells 1·10 mg. In tetany these drop to the average figures: whole blood 5·7 mg., plasma 5·26, and cells 0·46. These figures show a loss of calcium amounting to 27·2 per cent. for whole blood, to 54·4 per cent. for cells, and to 35·2 per cent. for the plasma.

(2) Diffusible calcium in normal serum averages from 60 to 70 per cent., while in severe parathyroid tetany it amounts to 94 per cent. of the total calcium.

(3) While there is an immediate state of alkalosis following parathyroidectomy, this condition is not necessarily marked, and with the development of the signs of severe tetany it passes rapidly into a condition of acidosis as shown by a steadily falling pH of the blood.

(4) The immediate relief of the condition consequent upon the withdrawal of 70–100 c.c. of blood is indicative of a toxic causative factor.

(5) The calcium deficiency and the great loss of colloidal calcium are probably indicative of a rapid protein disintegration.

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THE EXPRESSION OF THE RESULTS OF THE WASSERMANN REACTION IN UNITS.

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IT has become the fashion nowadays to express biological reactions in units where the exact amount of the reacting substance cannot be determined gravimetrically. The advantage of such a procedure for recording the strength of the Wassermann reaction is at once apparent for the purpose of comparison with other laboratories and for noting the effects of treatment.

At present the Wassermann reaction is usually expressed in such nomenclature as negative, weak positive, positive, strong positive, etc., or as some numeral which indicates the degree of reaction in a particular dilution of the patient's serum. For instance, in the Fildes-McIntosh method (1913, 1918) the results are recorded by the figures 4, 3, 2, 1 or 0; 4 meaning a complete inhibition of hæmolysis; 3, 25 per cent. of hæmolysis; 2, 50 per cent.; 1, 75 per cent., and 0 complete hæmolysis. In the actual test 5 falling dilutions of patient's serum, 0·1 to 0·006 c.c., are used, so that if the reaction changes, say, from 44200 to 42000, we can appreciate that the amount of Wassermann substance is decreasing, but we do not know the amount of the decrease. Browning and Kennaway (1920) a short time ago suggested a criterion of positivity based upon a comparison of the amount of complement giving complete lysis with a serum and with the negative control. In France, Vernes' syphilimetric method (1920) is another attempt to obtain an interpretation of the serum reaction, which will give a better control of treatment.

The adoption of a unit system for the Wassermann reaction is not quite as easy as would appear, because it is not sufficient merely to multiply the serum dilution by some simple index. Such a procedure as this does not take into account the fact that in the Wassermann test different degrees of positivity may be obtained in the same tube, *i. e.* different degrees of hæmolysis. So that a unit system for the Wassermann must, in addition, take into account this possible variation in hæmolysis as well as the variation in the amount of serum.

The constant on which we have fixed for our unit system is the antigen. Previous researches have shown that the McIntosh-Fildes antigen (1913) is constant in its complement-fixing properties. But in addition to the antigen there are two other constituents of the Wassermann reaction which may be regarded as constants in that they are standardised before each test: (*a*) sensitised red cells, and (*b*) 2½ minimal hæmolytic doses of complement. The exact value of these constants can be best shown by examining each separately.

The antigen, as has already been indicated, shows no variation either in regard to specific fixation or inhibitory power, and so must be regarded as a constant. The sensitised red corpuscles are used solely as indicators of the presence of free complement. Slight variations in their susceptibility to hæmolysis and to the extent to which they are packed by the centrifuge does occur, but it must be small. To get the best results the red cells should be as fresh as possible, as keeping for a few days in the ice chest, and more especially if some preservative has been added, renders them more or less sensitive to hæmolysis Very occasionally red cells are obtained which show an increased resistance to hæmolysis. Such variations, however, will always be shown up in the preliminary standardisation. Lastly in the test proper the value of the $2\frac{1}{2}$ minimal hæmolytic doses of complement is undoubtedly a constant, particularly in relation to the 0·5 c.c. of sensitised red cells. The preliminary standardisation of complement determines its hæmolytic titre, and, in addition, insures that it does not behave in an abnormal manner in the presence of antigen, while any abnormal inhibition on the part of the serum to be tested will be shown up by the control tube which contains $1\frac{1}{4}$ M.H.D. of complement.

It has been stated that so variable are the results of Wassermann tests on the same serum that the reaction can be of little value for comparative tests, but the constancy of the Wassermann reaction based on the principles we have adopted (McIntosh, 1918) is greater than the experience of certain writers would allow. Provided the preliminary standardisation is accurately carried out constant results should follow. The results given below were obtained by testing the same pooled serum over a period of five months. The serum was placed in small vaccine bulbs and kept at −10° C. The results were judged by opacity in the ordinary routine method without any attempt at estimation of the hæmolysis by means of a colour scale.

25.1.23	4 4 4 4 3 1 0
1.2.23	4 4 4 4 4 1 0
8.2.23	4 4 4 4 1 0 0
8.3.23	4 4 4 4 2 0 0
15.3.23	4 4 4 4 3 0 0
31.5.23	4 4 4 4 1 – –

As already pointed out, it is the different degrees of specific adsorption in any single dilution of the serum as shown by degrees of hæmolysis corresponding to the figures 4, 3, 2 or 1 which complicates matters. As a preliminary to an elaboration of a unit system it was necessary to determine the specific fixation of the complement which corresponded to these degrees of hæmolysis from an estimation of the amount lost through nonspecific adsorption by antigen and by serum.

In order to do this a double set of experiments were set up to determine—

(1) Extent to which complement was fixed by antigen alone.

(2) Extent to which complement was fixed in the presence of antigen with a negative serum.

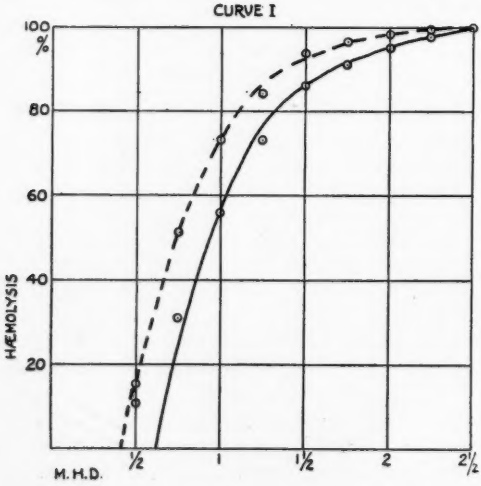
In each of the above a series of 10 tubes was taken. In the end tube was placed $2\frac{1}{2}$ M.H.D. of complement, and in the rest diminishing quantities down to one-tenth that amount, the actual quantities being 0·25 c.c. to 0·025 c.c.

To the first series was then added 0·25 c.c. of Wassermann antigen and then saline to bring the total volume to 0·5 c.c.; to the second series 0·25 c.c. of antigen + 0·05 c.c. of a negative human serum, the volume as before being brought up to 0·5 c.c.

TABLE I

		TUBES										
		1	2	3	4	5	6	7	8	9	10	
ANTIGEN	1	0	22	52	70	85	92	95	95	99	100	1/24
	2	0	15	57	85	90	96·5	97	98·5	100	100	1/28
	3	0	15	65	?90	?95	94	97·5	100	100	100	1/30
	4	0	10	30	65	77	92·5	96·5	100	100	100	1/16
TOTALS		0	62	204	220	252	375·0	386	393·5	399	100	
AVERAGE		0	15·5	57	73	84	93·7	96·5	98·4	99·7	100	
ANTIGEN & SERUM	1	0	9	26	61	74	90	92	97	99·5	100	
	2	0	10	30	63	74	87	90	94	97	99·3	
	3	0	24	49	67	77	85	90	93	96	99	
	4	0	3	20	43	69	83	91	98	100	100	
TOTALS		0	46	125	234	294	345	363	382	392·5	398	
AVERAGE		0	11	31	56	73	86	91	95	98	99·5	

CURVE I



Each series was incubated for 1 hour at 37°C., and at the end of that time 0·25 c.c. of sensitised red cells were added and the tubes placed in the water-bath for 20 minutes.

Results were read as percentage of hæmolysis by diluting each tube with saline, allowing to settle, and comparing with a standard scale prepared from the same blood.

Thus—

0	per cent.	=	Wassermann 4
25	,,	=	,, 3
50	,,	=	,, 2
75	,,	=	,, 1
100	,,	=	,, 0

Table I gives a composite chart determined from these two series of experiments. It is compiled from 8 antigen tests and 20 negative sera tests. The actual hæmolytic titre of the complement as estimated in the ordinary preliminary Wassermann standardisation is given in brackets at the side.

Curve I shows graphically these results and the effects of negative sera on the non-specific adsorption of complement. The full line represents the adsorption by antigen in the presence of negative sera, and the dotted line without serum. The amounts of complement corresponding to the different degrees of hæmolysis or readings in the *first tube* of the Wassermann can readily be determined, while the actual

amounts of complement (in units of M.H.D.), which must be fixed specifically to give these readings, is obtained by subtraction from $2\frac{1}{2}$ M.H.D.—the amount of complement used in the test.

TABLE II.

Wassermann test-reading.	Hæmolysis in percentage.	Specific adsorption of complement in M.H.D. units.
4	0	1·87
3	25	1·75
2	50	1·57
1	75	1·25
0	100	0·0

In the second and succeeding tubes the quantities of complement adsorbed will not be the same for similar readings of hæmolysis because the quantity of serum is decreasing. This, however, is easily calculated, and is shown on Table III.

TABLE III

W. R. READING	QUANTITY OF SERUM IN TUBE IN C.C.S	M. H. D COMPLEMENT TO BE FIXED	RECIPROCAL OF Q. OF SERUM	DOSES OF COMPLEMENT FIXED	= NEW WASSERMANN UNITS
44444	1/160	2·057		329·1	
44443		1·937		309·9	
44442		1·757		281·1	
44441		1·437		229·9	
44440	1/80	2·045		163·6	
44430		1·925		154·0	
44420		1·745		139·6	
44410		1·425		114·0	
44400	1/40	2·02		80·8	
44300		1·9		76·0	
44200		1·72		68·8	
44100		1·4		56·0	
44000	1/20	1·97		39·4	
43000		1·85		37·0	
42000		1·67		33·4	
41000		1·35		27·0	
40000	1/10	1·87		18·7	
30000		1·75		17·5	
20000		1·57		15·7	
10000		1·25		12·5	

In all these calculations it is necessary to assume that the non-specific adsorption of a positive serum is identical with that of a negative serum.

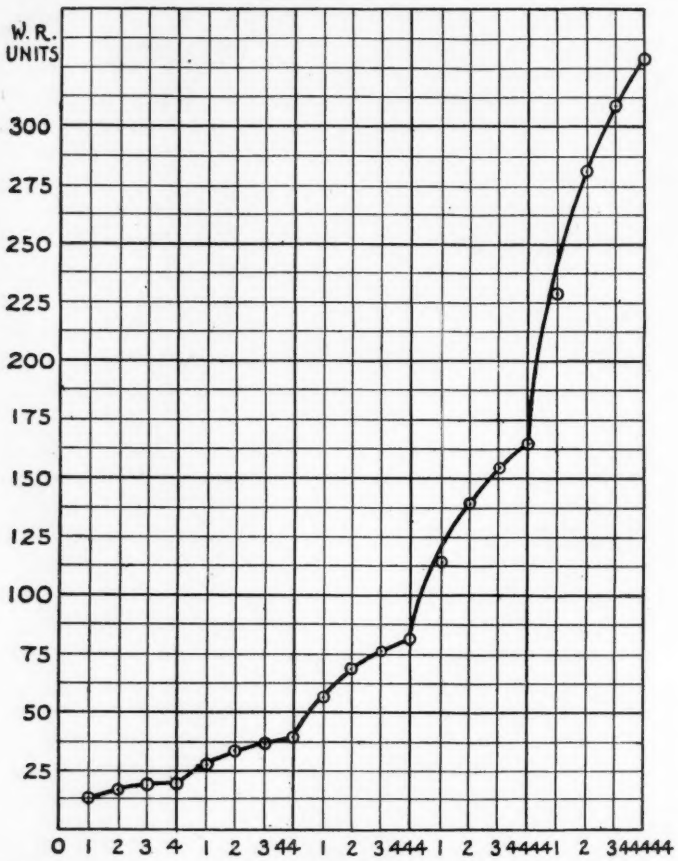
Contrary to a general belief, negative sera, therefore, are not protective, but 0·1 c.c. adsorbs about 0·2 of an M.H.D. of complement.

In the second tube of the Wassermann test we have only 0·05 of serum, which will adsorb, not 0·2, but 0·1 M.H.D. Hence for this tube, in estimating the M.H.D. fixed, we must add 0·1 of an M.H.D. to all the figures of column 3 of Table II. A similar correction must be applied for the remaining tubes.

As a suitable unit for the Wassermann reaction we propose the quantity of complement in M.H.D. fixed (specific fixation) multiplied by the reciprocal of

the fraction of a cubic centimetre of serum used in the last tube showing inhibition. The relation between such units and the old readings of the Wassermann is indicated in Curve II. From this diagram the unit value can be seen at a glance.

CURVE II.



It now remains to set a standard of positivity for the new units. It is usual to take a 3 reading in the first tube as indicative of syphilitic infection in a case without definite history. This in the unit system is equivalent to 17·5 units (*i.e.* complement fixed for 25 per cent. hæmolysis = 1·75 M.H.D. × reciprocal of fraction of c.c. of serum employed = $\frac{1}{\frac{1}{10}} = 10$ ∴ units = 1·75 × 10 = 17·5).

In a treated case, on the other hand, even a "1" is regarded as indicative of some remaining infection, and this figure is represented by $1{\cdot}3 \times 10 = 13$ units.

Curve II is based on this relationship, and from it the new unit value for any reading of the Wassermann can be determined directly.

The advantages of the unit system have already been commented on, particularly with regard to obtaining comparable results between different laboratories and obtaining a better indication of the effects of treatment. The application of the unit system would not interfere with the particular Wassermann technique used at particular laboratories. It would only mean that a standard serum of so many units strength and a standard antigen would have to be supplied. The method, however, can be applied directly to any method of carrying out the Wassermann reaction by reducing the number of M.H.D. of complement fixed to the required M.H.D. for a 5 per cent. suspension of sheep cells. The result from the laboratory would be given as positive, weak positive, negative, etc., with the units given in brackets at the side.

COMPARISON WITH THE UNIT OF THE SIGMA TEST OF DREYER AND WARD.

Quite recently we had an opportunity of comparing this proposed Wassermann unit with the unit system employed in the Sigma test of Dreyer and Ward (1921). A series of bloods were under examination on behalf of the Medical Research Council for the purpose of comparing the Wassermann and Sigma tests, and which will shortly be published.

Table IV gives an analysis of these results; on the left the ordinary Wassermann readings are given and on the right the Sigma results which corresponded. Plotting these results, W.R. units against Sigma units, a good line can be obtained when the Sigma averages are compared with the new

TABLE IV

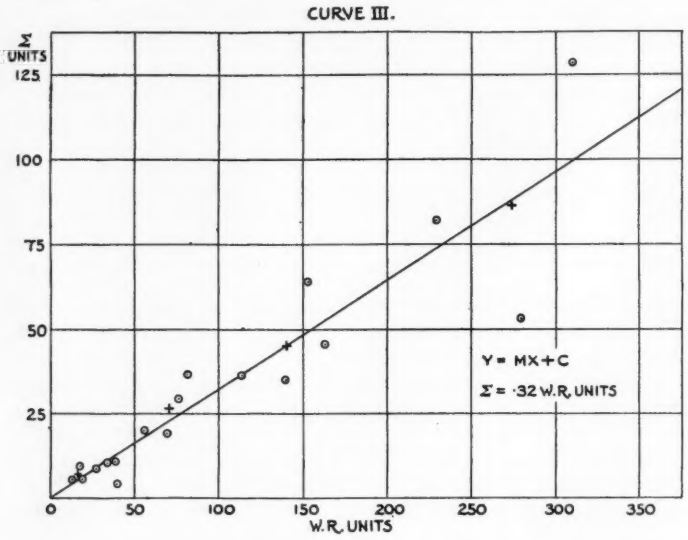
W. R. READING	NEW UNITS	GROUP AVERAGE	AVERAGE OF SIGMA READINGS	GROUP AVERAGE OF SIGMA READINGS
44444	329·1	273·6	128	87
44443	309·9		53	
44442	281·1		82	
44441	229·9			
44440	163·6	142·8	46	45·2
44430	154·0		64	
44420	139·6		35	
44410	114·0		36	
44400	80·8	70·4	37	26·4
44300	76·0		29·5	
44200	68·8		19	
44100	56·0		20	
44000	39·4	34·2	3·6	9·3
43000	37·0		10·2	
42000	33·4		10·0	
41000	27·0		8·3	
40000	18·7	16·1	5·3	7·08
30000	17·5		9·9	
20000	15·7		7·4	
10000	12·5		5·3	

Wassermann units (Curve III). A still better line, as perhaps is to be expected, is obtained if we plot out the group average of these units, these groups corresponding to the five tubes of the Wassermann reaction.

Solving this curve from the formula—

$$Y = MX + C$$

we find find that Sigma unit = 0·317 W.R. units.



Dreyer and Ward take as the diagnostic limit 1·63 units, which, according to the above formula, = 5·05 W.R. units.

Again, a W.R. "3," regarded as diagnostic, = 19 W.R. units or 19 × 0·317 = 6·3 Sigma units.

This shows that there is a discrepancy in the setting adopted as standard in these two tests. Either that of the Wassermann is too high or that of the Sigma too low.

The Wassermann, however, has now had many years of experience in diagnosis and in treatment, and the standard is built up on this basis. In the light of these researches the Wassermann standard may want some adjustment. However, clinical experience, particularly in watching the effects of treatment, and time can alone determine what the standard should be. Because the Sigma obtains slightly "better" results in cases under treatment, it has been assumed by certain workers that it is a more delicate test. This is not necessarily so, and, as the above figures show, the improvement probably depends on the basis adopted as the standard of specificity. Further, the close parallelism of the systems, as shown by the curves, suggests that the Wassermann reaction and syphilitic precipitation tests are produced by one and the same substance in spite of experiments to the contrary (Mackie, 1923).

CONCLUSIONS.

A unit system for the expression of Wassermann results is described above. The method is applicable to any technique of carrying out the Wassermann test by reducing the number of M.H.D. of complement fixed to the required M.H.D. for a 5 per cent. suspension of sheep cells, and multiplying this result by the reciprocal of the quantity in cubic centimetres of serum employed.

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AUTO-DISSOCIATION OF AGGLUTININ-ANTIGEN COMPLEX.

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THE compound formed between antigen and antibody has hitherto been supposed to be a fairly stable one, at any rate by those holding Ehrlich's views. A certain amount of work has been done in efforts to resolve this compound, so that the antibody may be procured free, so far as possible, from the other constituents of the serum from which it was obtained.

Notably, Huntoon (1921) has attempted to effect this release and recovery with some success; he used methods of salting out combined with alternations of freezing and warming and recovered a portion of his immune bodies.

The following property of the compound formed, which I discovered by following up an apparent error in technique, is, I believe, new to the literature.

Recently I have been testing the staphylococcal group for serological types and found it necessary to use absorption of agglutinin tests. The method was the usual one, consisting in absorption of a 1:25 dilution of serum by a suspension of 50,000 millions per 1 c.c. of the coccus to be tested with the usual controls. After incubating for two hours at 37° C. and centrifuging out the coccus, the supernatant serum was tested for remaining agglutinins against the homologous coccus.

On one occasion I was interrupted in a test at the point when the incubation had been completed, leaving the separation of the cocci to be done, and I therefore placed the cocci, still in contact with the diluted serum, to stand till the following day at laboratory temperature, as I had occasionally done before. I was prevented from returning to complete the test for some 72 hours, and when I then had separated the cocci and serum and finished the test I was surprised to find that none of the cocci, which included two homologous examples as controls together with six for testing, had absorbed any appreciable amount of agglutinin. I naturally thought that I had made some error and immediately repeated the test with the same materials, but taking only 24 hours over it, to find the homologous cocci, with two of those for testing, had absorbed the agglutinin completely, while four others had not done so at all.

On reviewing the previous unsatisfactory experiment I failed to find any flaw in the technique and determined to repeat it, allowing a pause of 72 hours to intervene between absorption and separation as before. The result was the same as in the former one. I repeated this with other sera and cocci of different types and always the agglutinins were found free at the end, the original titre of the serum being hardly altered, if at all.

Obviously the connection of antigen and agglutinin appeared to be of an evanescent nature under the conditions I was using, but the dissociation did not take place within 48 hours of absorption. Evaporation did not cause it, as the tubes were tightly sealed, and lost only a very small fraction, if anything, during the waiting period. The menstruum used throughout was normal saline (0·85 per cent. NaCl, alcohol purified, in glass-distilled water).

The following experiment was then put up to settle the point:

A quantity of 1 in 25 dilution of a serum (No. 46) was made, and suspensions of the homologous No. 46 coccus of 50,000 millions and 100,000 millions per 1 c.c. These two strengths were used in case it might be thought that the smaller dose, though hitherto apparently sufficient, was not in reality large enough to absorb all agglutinin. Half of the serum dilution was mixed with an equal quantity of the lower strength suspension and the other half with the same quantity of the higher one. After incubation at 37° C. for two hours and a thorough shaking, one third of each mixture was put aside to wait for 96 hours at laboratory temperature, while the remaining portions were centrifuged at high speed till the cocci were completely sedimented, to eyesight. The fluids were then carefully removed and half of each were tested immediately in the usual manner, while the other halves were put to stand by the side of the serum and coccal mixtures for 96 hours. The protocol (see Table I) shows the result.

It will be seen that the agglutinin has been completely absorbed by both strengths of coccal suspension in the portion tested immediately, but it is otherwise in the portions left to stand for 96 hours in contact with the cocci. The serum in this case has received back its agglutinin to its full strength apparently. The serum, on the other hand, which was separated from the coccus, as might be expected, still showed the absence of agglutinins, though it is true there was a trace shown at the 1 in 100 dilution, with the weaker dose of cocci. These traces are difficult to account for, and may be due to a variety of causes. I think the explanation in this case probably is that it is

very difficult to remove the last cocci from a suspension, and although apparently clear the fluid may still contain nearly 100 million cocci per 1 c.c. These cocci, should they still remain in the fluid, might well give up their fraction of agglutinin on standing, hence possibly the "Trace."

TABLE I.

Absorbing dose. No. 46 coccus.	Unabsorbed serum. 1 in— 100	200	400	800	Absorbed serum. 1 in— 100	200	400	800	Remarks.
Immediate test.									
50,000 mill.	++	++	+	(+)	−	−	−	−	Complete absorption of agglutinins.
100,000 mill.					−	−	−	−	
Tested after 96 hours' wait.									
50,000 mill.	++	++	+	(+)	tr.	−	−	−	Test of serum, separated from cocci, after 96 hours' wait. Almost complete absorption.
100,000 mill.					−	−	−	−	
50,000 mill.	++	++	+	(+)	++	++	+	(+)	Left in contact with cocci. No absorption.
100,000 mill.					++	++	+	(+)	

++ = complete agglutination. + = obvious agglutination, fluid not clear. (+) = agglutination only visible with × 8 lens.

The result of this experiment, I think, shows irrefutably that agglutinin, after absorption, dissociates itself from its antigen after standing at normal temperature for a sufficient time.

The next experiment was to ascertain whether dissociation would occur in normal saline alone. No agglutination, however, occurred after allowing the absorbing cocci to stand in saline for 96 hours, the cocci having previously been well washed free of serum.

A similar experiment to the last was now tried, substituting normal rabbit serum (serum, both normal and immune, was always from the rabbit). This showed no agglutination previously with the same coccus used for the experiment with plain saline. The result was not so good as with the immune serum, for although there was some agglutinin in the serum when tested after its 96 hours' contact with the absorbing coccus, the titre barely amounted to half that of the original immune serum. Partial dissociation, however, had occurred.

The next experiment was undertaken to see whether agglutinin could be transferred by this method from one immune serum to another, though heterologous, immune serum.

Two serologically different cocci were taken with their homologous sera—No. 20, a Type I *St. pyogenes*, and No. 46, a Type II *St. epidermidis albus*. The normal agglutinations of these were as follows:

	1 in 100	200	400	800
No. 20 serum *v.* No. 20 coccus	++	++	+	+
No. 20 ,, *v.* No. 46 ,,	−	−	−	−
No. 46 ,, *v.* No. 46 ,,	++	+	+	(+)
No. 46 ,, *v.* No. 20 ,,	(+)	−	−	−

Both cocci gave no agglutination with 1 : 100 normal serum.

A dilution of No. 46 serum was put up in the usual manner to absorb with No. 46 coccus. The coccus, then, after centrifugalisation and well washing twice in saline, was added to a dilution of No. 20 serum (heterologous). This mixture and the centrifuged off No. 46 serum were then put to stand at room temperature for 96 hours, and then, after centrifuging the No. 46 coccus from the No. 20 serum, tested in the usual way for both agglutinins.

		Serum dilution.			
		1 in—			
		100	200	400	800
No. 20 serum after 96 hours	*v.* No. 20 coccus	+	+	+	+
No. 20 ,, ,,	*v.* No. 46 ,,	++	+	+	+
No. 46 ,, (absorbed)	*v.* No. 46 ,,	+	(+)	−	−
No. 46 ,, ,,	*v.* No. 20 ,,	+	(+)	−	−

It will be observed that the No. 46 serum which was absorbed by its homologous coccus is very much reduced in strength for its own coccus, but all agglutinin is not quite absorbed, probably for the reason given above. For No. 20 coccus, however, it is also raised in titre to a small extent, the explanation of which I am not able to suggest.

The No. 20 serum, however, which has been in contact with the absorbing coccus for 96 hours, has lost a small fraction of its agglutinin for its own coccus, but has gained agglutinin for No. 46 coccus, which was entirely absent before, almost up to the full titre of the No. 46 serum.

The agglutinin therefore seems to be transferable from one immune serum to another in the manner shown.

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THE COMPLEMENT-FIXATION TEST IN THE DIAGNOSIS OF GONOCOCCAL INFECTION IN WOMEN.

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The method adopted in carrying out the test was essentially that advocated by L. W. Harrison. Two types of antigen were used, one made up following exactly the technique of D. Thomson (1918), and another prepared by a method described below, parallel observations being made with each. In some cases, also, parallel tests were made with the gonococcal antigen prepared by D. Thomson and put on the market by Genatosan, Limited.

A powerfully deviating serum was used to compare the strength of different antigens, and in all deviation tests as a positive control.

Tests were made on sera taken from 172 cases attending the women's V.D. clinics of the Elizabeth Garrett Anderson and the Royal Free Hospitals, care being taken to include as far as possible all stages and manifestations of the disease.

METHODS.

Preparation of a Strongly Deviating Serum.

A rabbit was injected at weekly intervals with increased doses of a gonococcal emulsion made from 24-hour cultures of eight different strains of gonococci, grown on a serum-agar medium of pH 7·9.

After a dose of 6000 million gonococci had been reached a serum was obtained which in a 1/1500 dilution deviated three doses of complement completely, and in a 1/6400 dilution gave agglutination with an emulsion made from those strains of organisms which had been used for immunisation.

Preparation of Antigens.

The method used was a modification of that described by Altmann and Schultz (1909), with reference to the typhoid bacillus. A standard emulsion of gonococci—made from 24-hour cultures of eight different strains—was made, such that 1 c.c. contained 3000 million organisms. An equal volume of 4 per cent. commercial antiformin was added and the resulting solution heated to 56° C. for 45 minutes. At the end of this time a perfectly clear solution was obtained, in which absence of free chlorine was proved by testing with starch-iodide paper.

The solution was titrated back to neutral with decinormal acid, the point taken as "neutral" being that at which no colour was given with phenolphthalein and a definite purple tint with bromo-cresol-purple (*i. e.* between pH 6·8 and pH 8·3). Normal saline containing 0·5 per cent. carbolic acid was added till the final volume was three times that of the original emulsion. The resulting solution, containing 1000 million dissolved gonococci per c.c., was used as a stock antigen. This will be referred to as Antigen A.

A second antigen was made from the same eight strains, carrying out in every detail the technique described by Thomson. This, which also contains 1000 million of dissolved gonococci per c.c., will be referred to as Antigen B.

Technique Employed in Carrying Out the Test.

1. Inactivation and dilution of the serum to be tested: Inactivation was performed by heating in a water-bath at 55° C. for 15 minutes.

Experiments made with human sera, in dilutions of 1/2, 1/5 and 1/10, showed that the 1/2 dilution, after inactivation, was liable to deviate complement too frequently for its use to be practicable. 1/5 was found to be the lowest dilution in which the serum was not anticomplementary; this solution with 0·85 per cent. saline was therefore used habitually.

2. Titration of complement against red cells in the presence of antigen: To 8 Wassermann tubes was added 0·1 c.c. of fresh guinea-pig serum (complement) in the following dilutions:—

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
1/10	1/15	1/20	1/30	1/40	1/60	1/80	1/120

To each was added 0·1 c.c. of the stock antigen diluted 1/5 in the case of Antigen A, 1/10 for Antigen B.

To each again was added 0·1 c.c. of normal saline, to make the volume the same as that in the test itself. As control a second series of tubes was put up, containing complement diluted as before, to each being added 0·2 c.c. of saline. In this way any anticomplementary action on the part of the antigen itself could be detected.

The tubes were shaken, placed in the ice-chest for one hour, and then transferred to the water-bath at 37° C. for half-an-hour. To each was then added 0·1 c.c. of a 2·5 per cent. suspension of sensitised sheep corpuscles. The tubes were then replaced in the water-bath for 15 minutes and the reading taken at the end of that time.

The greatest dilution of complement producing complete hæmolysis in the series containing antigen was taken as the minimum hæmolytic dose (M.H.D.), this factor being determined before every experiment. The M.H.D. in the series without antigen was generally found to be less than this; a complement working at 1/30 with antigen would hæmolyse at 1/40 with saline. Any antigen deviating complement to a greater degree than this was rejected as too anticomplementary for use.

3. The test itself: For each serum to be tested, four tubes (I, II, III and IV) were used.

To each of the four was added 0·1 c.c. of the inactivated serum diluted 1/5.

To I was added 0·1 c.c. of complement, containing 1 M.H.D.
To II ,, ,, ,, ,, 1 ,,
To III ,, ,, ,, ,, 2 ,,
To IV ,, ,, ,, ,, 3 ,,

To II, III and IV were added 0·1 c.c. of antigen diluted 1/5 for Antigen A, or 1/10 for Antigen B. To I was added instead 0·1 c.c. of saline, this tube acting as a serum control. Parallel with tubes II, III and IV were put up

three tubes containing antigen and complement as in II, III and IV, but in which the serum was omitted and replaced by 0·1 c.c. of saline; these formed the antigen control.

The tubes were placed in the ice-chest overnight. Next morning 0·1 c.c. of a 2·5 per cent. suspension of sensitised sheep-cells was added, and the reading taken after 15 minutes in the water-bath at 37° C.

The serum control tube I and the antigen control tubes should show complete hæmolysis. Known positive and negative sera were always included in a series of tests, and the results of an experiment were accepted only if all the controls were satisfactory.

EXPERIMENTS TO ESTIMATE AND COMPARE THE PROPERTIES OF ANTIGENS.

1. *The greatest strength in which the antigens could be used* was determined by titrating a constant volume of each antigen in dilutions of 1/2, 1/5 and 1/10, against varying dilutions of complement, after the method already described. It was found that both antigens in a 1/2 dilution were strongly anticomplementary.

Antigen A diluted 1/5 was only slightly anticomplementary, permitting hæmolysis with a complement dilution of 1/30, the complement used alone giving a M.H.D. of 1/40.

Antigen B diluted 1/5 gave with this same complement a M.H.D. of 1/10, and was therefore rejected in this strength.

In a 1/10 dilution it deviated complement only to the same extent as the 1/5 dilution of Antigen A.

Antigen A could therefore be used at double the strength found possible for Antigen B.

The gonococcus antigen supplied by Genatosan Limited was found to be identical with Antigen B as regards its anticomplementary action.

2. *Comparison of the antigenic power of antigens.*—Each antigen in its appropriate dilution was put up with a series of increasing dilutions of the antigonococcal rabbit serum, 3 M.H.D. of complement being added in each case.

It was found that, using Antigen A 1/5, complete deviation was given by the serum in a 1/1500 dilution. With Antigen A 1/10, the highest dilution of serum giving complete deviation was 1/900; with Antigen B 1/10 partial deviation was given by the same dilution of serum.

Using the commercial antigen, no deviation was obtained in a serum dilution of more than 1/200; this, however, may well be explained by the fact that the commercial antigen was non-specific to the serum, whereas Antigens A and B were both made from the strains used in preparation of the serum.

3. *Effect on antigens of titrating them to varying degrees of hydrogen-ion concentration.*—A series of antigens was made as previously described, but the final reaction was titrated to a definite pH, using phenol-red as indicator, and matching to standard phosphate solutions of known pH.

It was found that both Antigens A and B at pH 7·0, 7·4 and 7·8 showed no appreciable difference as regards either their anticomplementary or antigenic properties. When brought to a pH as alkaline as 8·2 both became slightly more anticomplementary. It seemed therefore that in the rough

titration to " neutral " of the standard antigens, the variations of pH possible within the range of experimental error were of negligible importance.

4. *Anticomplementary effect of too prolonged fixation at low temperatures.* —Griffith and Scott (1920) recommend that a serum to be tested should be left in contact with complement and antigen for some hours at 0° C. before the sensitised red cells are added, claiming that a more thorough fixation of complement is obtained by this means.

It was noticed on several occasions that the antigen controls treated in this way became anticomplementary to doses of complement with which, in the preliminary titrations, they had allowed complete hæmolysis to occur. A series of tubes was therefore put into the ice-chest, each containing the same amount of antigen with one M.H.D. of complement, a tube being withdrawn and tested with sensitised red cells at 2-hourly intervals. It was found that complete hæmolysis was obtained up to the fifteenth hour. After 16 hours there was slight deviation of complement; at 18 hours deviation was complete.

In performing the test, therefore, 15 hours was the time allowed for fixation in the ice-chest, with satisfactory results.

RESULT OF 172 EXPERIMENTS ON SERA TAKEN FROM PATIENTS ATTENDING A WOMEN'S V.D. CLINIC.

The following charts give the results of the test with an analysis of the cases examined.

TABLE I.

	Total number examined.	Doses of complement deviated.						Deviation positive.		Valid positives (1 M.H.D. or more).	
		1/2-1	1	2	3	4	5	Total.	%	Total.	%
I.—G. cases:											
1. Pathologically proved	35	13	12	2	—	1	—	28	80	15	42·8
2. Clinically typical	44	16	14	—	—	—	—	30	68	14	31·8
II.—Cases not suggestive of G., but giving a positive Wassermann reaction	52	14	5	—	—	—	—	19	36·5	5	9·6
III.—Negative control cases, suggestive of neither G. nor S.	41	3	—	—	—	—	—	3	7	0	0·0

TABLE II.

Analysis of the 79 G. Cases in Group I of Table I.

	Number examined.	Deviation positive.	Deviation negative.
1. Acute urethritis or endocervicitis, *i. e.* within 4 weeks of onset	13	7	6
2. Chronic urethritis or endocervicitis	34	25	9*
3. Reinfection in old cases	6	6	0
4. Acute salpingitis	2	1	1
5. Chronic salpingitis	7	6	1
6. Bartholin's abscess	6	4	2
7. Vulvo-vaginitis of children	6	5	1
8. Arthritis at some stage of the disease	5	4	1

* Four of these cases were regarded as clinically cured.

TABLE III.

Comparison of Antigens A and B in 55 Cases giving Deviation with Each.

	Slight hæmolysis.	About 50 per cent. hæmolysis.	Nearly complete.	Complete to 1 M.H.D.
Antigen A	4	13	12	26
Antigen B	6	18	10	21

It will be seen that, of the 79 cases regarded as genuine gonococcal infections, 58 (73 per cent.) gave a positive result. Of these, however, 55 deviated no more than 1 M.H.D. of complement; 26 completely and 29 partially, using Antigen A; and 21 completely and 34 partially, using Antigen B. (See Table III.)

Of the remaining three cases, two deviated 2 M.H.D. complement completely; one showed complete deviation on two occasions with 4 M.H.D. Details of these three cases are given:

Case "1826." History of acute urethritis about 6 months previous to the taking of test. Bartholini abscess opened 6 weeks ago. Attack of acute salpingitis 3 weeks ago from which patient is recovering. Gonococcus grown in culture and isolated from both urethra and cervix. Deviation given to 2 M.H.D. (complete).

Case "1765." Acute urethritis and endocervicitis 5 weeks before test taken. Gonococcus grown in culture and isolated from urethra. Deviation to 2 M.H.D. (complete).

Case "1680." Chronic urethritis and endocervicitis of 18 months' duration. Arthritis affecting both knee-joints in the first months after infection. Gonococcus found repeatedly in film and culture. Deviation to 4 M.H.D. (complete).

In the attempt to obtain a higher deviation, a small number of sera were tested against their specific antigens, the gonococcus isolated from the patient being in each case made up to the same strength as the stock antigens. It was found that case "1765," which deviated 2 M.H.D. with the stock antigen, now deviated 4 M.H.D. Of five other cases, each giving complete deviation to 1 M.H.D. with the stock antigen, two now deviated 3 M.H.D., two deviated 2 M.H.D., and one remained unchanged in its deviating power.

Reference to the chart will show that the large number of positive sera in Group II deviated in every case 1 M.H.D. or less of complement. Here, too, the antigens gave very comparable results, Antigen B frequently giving a partial deviation where Antigen A gave complete deviation, but neither failing to show in some degree a deviation given by the other.

The three non-gonococcal and non-syphilitic cases entered as positive gave partial deviation only to 1 M.H.D. of complement.

CONCLUSIONS.

The results obtained establish the fact that a certain degree of deviation is produced in the majority—73 per cent.—of cases regarded as gonococcal, deviation being most constantly shown, as might be expected, in those cases where infection is of some weeks' standing, and particularly where the disease has passed beyond the purely local stage.

The low degree of deviation obtained in all but three of the series seems, however, to be a serious drawback to the adoption of the test as a means of diagnosis. The margin left for experimental error must necessarily be small

in dealing with such fine shades of deviation as that to a fraction of a single dose of complement.

Moreover, the fact that a high percentage of presumably non-gonococcal cases give positive results with this reaction and with the Wassermann reaction introduces another large factor of possible error.

It seems, however, that the complement-deviation test for the gonococcus may be of definite value, in cases where the Wassermann reaction is negative, *if deviation to less than* 1 *M.H.D. is disregarded.* As shown by Table I, rejection of smaller degrees of deviation would eliminate the errors of the negative control cases in Group III, and would reduce that of the syphilitic cases of Group II from 36 per cent. to 10 per cent., leaving 37 per cent. of positive cases in Group I. *The deviation to at least one complete dose given by this residue can be regarded as a definite entity reasonably free from error, and should be taken as the minimum in which confidence can be placed in using the test for diagnostic purposes.*

It should be remembered that there is no room for experimental error in working with deviations within such narrow limits; that the technique should be adhered to rigidly in every detail, and the carrying out of the test kept in the hands of experienced workers. *If these precautions are observed, a positive result may be taken as a definite aid in diagnosis.*

As regards an antigen for general adoption, the Antigen A made by the antiformin method here described has given consistently in these experiments slightly the more satisfactory results. It is not difficult to prepare, and it has shown no deterioration after being kept for three months at a temperature of 0°C.

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